THE UNIVERSITY *of* EDINBURGH

The Role of 11ß-Hydroxysteroid Dehydrogenase in Controlling Foetal Glucocorticoid Exposure

Rafn Benediktsson

Doctor of Philosophy

The University of Edinburgh

February 1995

To Hildur and Salvör

ACKNOWLEDGEMENTS

My deepest and most sincere thanks go to both of my supervisors, Dr. Jonathan R. Seckl and Professor Christopher R.W. Edwards, for their continued support, encouragement and enthusiasm about data which often seemed as dense as the Har.

I thank those that supported my research financially; the Scottish Hospital Endowments Research Trust, the Medical Research Council, the Sir Stanley and Lady Davidson Medical Research Fund, and the Wellcome Trust.

I thank those mentioned in the text who performed laboratory assays. Mrs. June Noble deserves special thanks for her excellent technical assistance and hard work with not only the *in-vitro* 11ß-HSD bioassay, but also with many other procedures. Midwifes and antenatal clinic staff at the Simpson Memorial Maternity Pavilion, without whose help this project wouldn't have been possible, also get special thanks. In addition, a great number of other people collaborated, discussed, criticised, supported, and helped me in a number of ways. Thus I thank the team at the Western General Hospital, especially Dan Burt, Robbie Lindsay, Brent Williams, Jill Smith, staff at the Biomedical Research Centre and the Medical Physics Workshop, not forgetting those sharing an office with me. Also, at the Centre for Reproductive Biology here in Edinburgh, Professor Andrew A. Calder, Harry Boyle and Rose Leask, and finally Professor Barker's team in Southampton.

ABSTRACT

Recent epidemiological data have implicated prenatal events in the development of cardiovascular disorders. Thus low birth weight strongly predicts the later occurrence of hypertension, type II diabetes mellitus, syndrome X and deaths from ischaemic heart disease. The mechanism linking prenatal events and later disease is not clear, although maternal malnutrition has been advocated. We have advanced the hypothesis that glucocorticoids might be important as they retard foetal growth and programme offspring hypertension in rats. The foetus has been thought to be protected from the 2-10 times higher maternal glucocorticoid levels by the placental enzyme 11ß-hydroxysteroid dehydrogenase (11ß-HSD), which is present in many tissues and in humans catalyses the conversion of the active glucocorticoid cortisol to inert cortisone (corticosterone to 11-dehydrocorticosterone in rats). The precise role of 11ß-HSD as a barrier to maternal glucocorticoids during prenatal life has not been fully characterised. The role of 11ß-HSD in controlling prenatal glucocorticoid exposure in humans and animals has thus been examined. Two isoforms of 11ß-HSD exist, type 1, a widespread NADP dependent reversible enzyme and type 2, a high affinity NAD dependent dehydrogenase found mainly in placenta and kidney.

11ß-HSD was found in abundance in the ovary and placenta. The main site of immunohistochemical staining and expression of mRNA (11ß-HSD-1) in the rat ovary was in the oocyte. 11ß-HSD was oxidative, inactivating corticosterone. In both rat placenta *in-vitro* (11ß-HSD-2), and human placenta *in-vitro* and *ex-vivo* (11ß-HSD-2) the bioactivity was also predominantly oxidative. The lowest placental enzyme activity at term (and hence the greatest foetal glucocorticoid exposure) was found in the smallest rats with the largest placentas, i.e. those in human studies who would be predicted to develop the highest adult blood pressures (birth weight vs. placental 11ß-HSD activity: $n = 56$; $r = 0.46$; $p < 0.0005$).

A method to examine 11ß-HSD function in fresh intact human placentas was developed (*ex-vivo* dual circuit cotyledon perfusion) which allows close

approximation to the *in-vivo* situation. The majority of cortisol, from low to high nanomolar concentrations, infused through the maternal circulation was metabolised to inert cortisone by the time it reached the foetal circulation, although considerable individual variation was observed. 11ß-HSD was the only significant contributor to placental cortisol metabolism at physiological maternal concentrations and inhibition of 11ß-HSD with either the liquorice constituent glycyrrhetinic acid or its hemi-succinate, carbenoxolone, resulted in abolition of the glucocorticoid barrier, allowing maternally administered cortisol to pass unmetabolised through the placenta. In a prospective study, on 16 normal primiparous women whose placentas were studied with this technique, a positive and significant correlation was found between the effectiveness of 11ß-HSD and offspring birthweight ($r = 0.67$; $p < 0.005$).

The relationship between placental 11ß-HSD effectiveness *in-vivo* and term cord blood osteocalcin (a sensitive marker of glucocorticoid exposure) was prospectively examined in 19 women. Cord blood levels of the bone specific protein osteocalcin were determined with radioimmunoassay. The lowest cord blood osteocalcin levels were found in the foetuses whose placental 11ß-HSD barrier function was poorest ($r = 0.58$; $p < 0.02$), (and had presumably had the greatest glucocorticoid exposure), suggesting that term cord blood osteocalcin levels might be a useful predictor of hypertension, ischaemic heart disease and possibly metabolic bone disease.

The findings presented in this thesis represent direct evidence that 11ß-HSD is the barrier to maternal glucocorticoids, its effectiveness correlating with foetal growth in rats (*in-vitro*), in humans (*ex-vivo*), and *in-vivo* with human cord blood osteocalcin levels (osteocalcin may be a marker of glucocorticoid exposure). In the light of studies on pregnant rats in which administration of exogenous glucocorticoids or 11ß-HSD inhibitors reduces birth weight and programmes hypertension in the offspring, it is reasonable to propose that increased foetal glucocorticoid exposure consequent upon attenuated placental 11ß-HSD function may play a role in intrauterine programming of later hypertension.

TABLE OF CONTENTS

DECLARATION 1

ACKNOWLEDGEMENTS 2

ABSTRACT 3

LIST OF FIGURES AND TABLES 9

ABBREVIATIONS 11

PUBLICATIONS FROM THIS THESIS 13

1. INTRODUCTION 16

1.1. EARLY DEVELOPMENT AND DISEASE 16

1.1.1. EPIDEMIOLOGY 16

1.1.2. DETERMINANTS OF BIRTH WEIGHT 21

1.1.3. PROGRAMMING 24

1.2. HORMONES 28

1.3. CORTICOSTEROID HORMONES 29

1.3.1. SYNTHESIS 29

1.3.2. REGULATION 33

1.3.3. METABOLISM 36

1.3.4. ROLE 37

1.4. 11ß-HYDROXYSTEROID DEHYDROGENASE 40

1.4.1. SYNDROME OF APPARENT MINERALOCORTICOID EXCESS 41

1.4.2. LIQUORICE AND THE KIDNEY 43

1.4.3. ISOFORMS OF 11ß-HSD 46

1.4.4. 11ß-HSD BEYOND THE KIDNEY 50

1.4.5. 11ß-HSD IN PREGNANCY 54

1.5. PLACENTAL ANATOMY 59

1.6. SUMMARY AND AIMS OF THE THESIS 63

2. MATERIALS AND METHODS 66

2.1. FLUID MEDIA 66
2.2. PREPARATION OF RADIOLABELLED MATERIALS 66
2.2.1. TRITIATED STEROIDS 66
2.2.2. IODINATED STEROIDS 67
2.3. RADIOIMMUNOASSAYS 69
2.3.1. CORTISOL 69
2.3.2. CORTISONE 70
2.3.3. OSTEOCALCIN 71
2.4. IN-VITRO ENZYME ASSAYS 71
2.4.1. 11ß-HYDROXYSTEROID DEHYDROGENASE 72
2.4.2. ALKALINE PHOSPHATASE AND N-γ-L GLUTAMYL ß-NAPHTHYLAMINIDASE 75
2.5. IMMUNOHISTOCHEMISTRY 76
2.6. IN-SITU HYBRIDISATION 76
2.7. NORTHERN BLOTTING 77
2.8. EX-VIVO PLACENTAL COTYLEDON PERFUSION 78
2.8.1. GENERAL METHODS AND EQUIPMENT SETUP 78
2.8.2. MONITORING OF THE PERFUSION 81
2.8.3. COMPARISON OF RESULTS AND SAMPLE PREPARATION 85

3. RAT OVARIAN 11ß-HSD 88

3.1. INTRODUCTION 88
3.2. METHODS 89
3.3. RESULTS 90
3.3.1. IMMUNOHISTOCHEMISTRY 90
3.3.2. mRNA EXPRESSION 91
3.3.3. BIOACTIVITY 92
3.4. DISCUSSION 96

4. RAT PLACENTAL 11ß-HSD 101

4.1. INTRODUCTION 101
4.2. METHODS 103
4.3. RESULTS 104
4.3.1. BIOASSAY EVALUATION 104
4.3.2. RELATIONSHIP OF 11ß-HSD TO RAT FOETAL GROWTH 108
4.3.3. IMMUNOHISTOCHEMISTRY AND MRNA EXPRESSION 112
4.4. DISCUSSION 115

5. HUMAN PLACENTAL 11ß-HSD IN-VITRO 122

5.1. INTRODUCTION 122
5.2. METHODS 124
5.2.1. SUBJECT SELECTION AND SAMPLE COLLECTION 124
5.2.2. ASSAYS 124
5.3. RESULTS 125
5.3.1. 11ß-HSD BIOASSAY EVALUATION 125
5.3.2. BIRTH WEIGHT AND HUMAN 11ß-HSD IN-VITRO 129
5.4. DISCUSSION 134

6. HUMAN PLACENTAL 11ß-HSD EX-VIVO 139

6.1. INTRODUCTION 139
6.2. METHODS 141
6.2.1. SUBJECT SELECTION 141
6.2.2. PERFUSION SYSTEM 142
6.2.3. SAMPLING AND ANALYTICAL METHODS 143
6.3. RESULTS 144
6.3.1. VALIDATION AND VIABILITY OF THE PREPARATION 144
6.3.2. THE BARRIER ROLE OF HUMAN PLACENTAL 11ß-HSD 153
6.3.3. EX-VIVO HUMAN PLACENTAL 11ß-HSD ACTIVITY AND BIRTH WEIGHT 160
6.3.4. HUMAN PLACENTAL 11ß-HSD ACTIVITY EX-VIVO, ALCOHOL & SMOKING 163

6.4. DISCUSSION 165

7. HUMAN FOETAL OSTEOCALCIN AND PLACENTAL 11ß-HSD 172

7.1. INTRODUCTION 172
7.2. METHODS 174
7.2.1. SUBJECT SELECTION AND SAMPLING 174
7.2.2. ASSAYS AND DATA ANALYSIS 175
7.3. RESULTS 176
7.4. DISCUSSION 178

8. RÉSUMÉ 181

9. BIBLIOGRAPHY 188

LIST OF FIGURES AND TABLES

Figure 1.1 Imprinting of Hypertension In-Utero and its Self Amplification 19

Figure 1.2 Basic Structure of Steroids. 28

Figure 1.3 Biosynthesis of Adrenocortical Steroids 32

Figure 1.4 A Schematic Outline of the Hypothalamic-Pituitary-Adrenal Axis 35

Figure 1.5 Metabolism of Cortisol 37

Figure 1.6 11ß-Hydroxysteroid Dehydrogenase 41

Figure 1.7 Inhibitors of 11ß-HSD 44

Figure 1.8 Enzyme Mediated Receptor Protection 46

Figure 1.9 The Human Placenta 60

Figure 1.10 The Rat Placenta 61

Figure 2.1 High Performance Liquid Chromatography Setup 74

Figure 2.2 The Perfusion Chamber. 79

Figure 2.3 Setup Diagram 80

Figure 3.1 Rat Ovary: Northern Blotting. 91

Figure 3.2 Rat Ovarian Bioactivity I: Time Course 93

Figure 3.3 Rat Ovarian Bioactivity II: Protein Concentration. 93

Figure 3.4 Immunohistochemistry of a Rat Ovary 94

Figure 3.5 Rat ovary In-Situ Hybridisation 95

Figure 4.1 Cofactor Preference of Rat Placental 11ß-HSD In-Vitro 105

Figure 4.2 Rat Placental 11ß-HSD In-Vitro Assay 106

Figure 4.3 Rat Placental 11ß-HSD Inhibition In-Vitro 106

Figure 4.4 Storage of Rat Placental Homogenate 107

Figure 4.5 Zonal Distribution of 11ß-HSD Bioactivity 109

Figure 4.6 Term Rat Foetal Weight and 11ß-HSD Bioactivity 110

Figure 4.7 Term Rat Placental Weight and 11ß-HSD Bioactivity 111

Figure 4.8 Rat Placental 11ß-HSD Activity, Term Foetal and Placental Weight 112

Figure 4.9 Immunohistochemistry of Term Rat Placenta 113

Figure 4.10 Term Rat Placenta In-Situ Hybridisation 114

Figure 5.1 Cofactor Preference of Human Placental 11ß-HSD In-Vitro 126

Figure 5.2 Human Placental 11β-HSD In-Vitro Assay: Fresh Trophoblast 127

Figure 5.3 Short Term Storage of Human Placental 11β-HSD 128

Figure 5.4 Human Placental 11β-HSD In-Vitro Assay: Stored Trophoblast 128

Figure 5.5 Human Birth Weight and 11β-HSD Bioactivity In-Vitro 130

Figure 5.6 Long Term Storage of Human Placental 11β-HSD 132

Figure 5.7 Repeat 11β-HSD Assay of Stored Human Trophoblast 133

Figure 6.1 Washout of Endogenous Glucocorticoids 145

Figure 6.2 Pattern of Cotyledon Lactate Washout/Production 145

Figure 6.3 Stability of 11β-HSD Activity in the Perfused Human Cotyledon 146

Figure 6.4 Light Microscopy Photomicrographs of Human Placenta 148

Figure 6.5 Electron Microscopy Photomicrographs of Human Placenta 149

Figure 6.6 Kinetics of ^{3}H-Cortisol Transfer and Metabolism During Perfusion 150

Figure 6.7 Details of Perfusate Extraction and Recovery of ^{3}H-cortisol 152

Figure 6.8 Elution Profiles of Cold Potential Cortisol Metabolites on HPLC 157

Figure 6.9 Typical HPLC Trace of a Foetal Effluent Perfusate Sample 157

Figure 6.10 Maternal Concentration and Transfer of Cortisol and Antipyrine 158

Figure 6.11 Corticosteroid Clearance Index and 11β-HSD Activity 158

Figure 6.12 Inhibition of Human 11β-HSD in the Perfused Placenta 159

Figure 6.13 The Effect of Carbenoxolone on Placental 11β-HSD Ex-Vivo 159

Figure 6.14 Human Birth Weight & Placental 11β-HSD Efficiency Ex-Vivo: A 162

Figure 6.15 Human Birth Weight & Placental 11β-HSD Efficiency Ex-Vivo: B 163

Figure 7.1 Term Cord Blood Steroid and Osteocalcin Levels 177

Figure 7.2 Mean Cord Osteocalcin and 11β-HSD Activity Index 177

Table 5.1 Human 11β-HSD In-Vitro: Study Details 129

Table 5.2 Human 11β-HSD In-Vitro: Pearson's Correlation Coefficients 130

Table 6.1 Human 11β-HSD Ex-Vivo: Subject and Study Details 161

ABBREVIATIONS

11ß-HSD	11ß-Hydroxysteroid Dehydrogenase
A	11-Dehydrocorticosterone
ACTH	Adrenocorticotrophic Hormone
Aldo	Aldosterone
allo-THF	5α-Tetrahydrocortisol
ALP	Alkaline Phosphatase
AME	Syndrome of Apparent Mineralocorticoid Excess
ANOVA	Analysis of Variance
ASD	Androstenedione
AVP	Arginine Vasopressin
B	Corticosterone
bp	Base-Pair
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
CBG	Corticosterone/Cortisol Binding Globulin
CBX	Carbenoxolone
cDNA	Complementary DNA
CNS	Central Nervous System
CRF	Corticotrophin Releasing Factor
cRNA	Complementary RNA
CV	Coefficient of Variation
DHEA	Dehydroepiandrosterone
DNA	Deoxyribonucleic Acid
DOC	11-Deoxycorticosterone
E	Cortisone
F	Cortisol
GALF	Glycyrrhetinic Acid Like Factor
GE	Glycyrrhetinic Acid

GI	Glycyrrhizic Acid
GNA	N-γ-L Glutamyl ß-Naphthylaminidase
HIV	Human Immunodeficiency Virus
HPLC	High Pressure/Performance Liquid Chromatography
IC_{50}	Concentration Achieving 50% Inhibition
IGF-1 and 2	Insulin Like Growth Factor 1 and 2
IGFBP-1 and 2	Insulin Like Growth Factor Binding Protein 1 and 2
IUGR	Intrauterine Growth Retardation
K_m	Michaelis Constant
KRB	Krebs-Ringer Bicarbonate Buffer
mRNA	Messenger RNA
NAD	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide - reduced form
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Nicotinamide Adenine Dinucleotide Phosphate - reduced form
POMC	Proopiomelanocortin
RNA	Ribonucleic Acid
SD	Standard Deviation
SEM	Standard Error of the Mean
SHR	Spontaneously Hypertensive Rat
TeBG	Testosterone Binding Globulin
THE	Tetrahydrocortisone
THF	Tetrahydrocortisol
TLC	Thin Layer Chromatography
tRNA	Transfer RNA
UTP	Uridine Triphosphate

PUBLICATIONS FROM THIS THESIS

Original Publications:

1. Teelucksingh S, Benediktsson R, Lindsay RS, Burt D, Seckl JR, Edwards CRW, Nan CL, Kelly R. Liquorice. *Lancet* 1991;337:1549.

2. Benediktsson R, Yau JLW, Low S, Brett LP, Cooke BE, Edwards CRW, Seckl JR. 11ß-hydroxysteroid dehydrogenase in the ovary: High expression in the oocyte. *J Endocrinol* 1992;135:53-58.

3. Benediktsson R, Lindsay RS, Noble J, Seckl JR, Edwards CRW. Glucocorticoid exposure in utero: new model for adult hypertension. *Lancet* 1993;341:339-341.

4. Edwards CRW, Benediktsson R, Lindsay RS, Seckl JR. Dysfunction of placental glucocorticoid barrier: link between fetal environment and adult hypertension? *Lancet* 1993;341:355-357.

5. Benediktsson R, Brennand J, Tibi L, Calder AA, Seckl JR, Edwards CRW. Fetal osteocalcin levels are related to placental 11ß-hydroxysteroid dehydrogenase activity in humans. *Clin Endocrinol* 1995; (In press).

6. Benediktsson R, Noble J, Calder AA, Edwards CRW, Seckl JR. The efficiency of the placental glucocorticoid barrier predicts birth weight in humans. (Submitted).

7. Langley-Evans SC, Phillips GJ, Benediktsson R, Gardner DS, Edwards CRW, Jackson AA, Seckl, JR. Protein intake in pregnancy, placental glucocorticoid metabolism and the programming of hypertension. (Submitted).

Reviews:

1. Edwards CRW, Walker BR, Benediktsson R, Seckl JR. Congenital and acquired syndromes of apparent mineralocorticoid excess. *J Steroid Biochem Molec Biol* 1993;45:1-5.

2. Benediktsson R, Edwards CRW. Apparent mineralocorticoid excess. *J Hum Hypertens* 1994;8:371-375.

3. Benediktsson R, Walker BR, Edwards CRW. Cellular selectivity of aldosterone action: role of 11-beta-hydroxysteroid dehydrogenase. *Current Opinion in Nephrology and Hypertension* 1995;4:(In press).

Abstracts:

1. Benediktsson R, Seckl JR, Edwards CRW. Placental 11ß-hydroxysteroid dehydrogenase: key role in controlling foetal environment? *J Endocrinol* 1992;132(Suppl):A85.

2. Campbell JC, Walker BR, Benediktsson R, Williams BC, Edwards CRW. 11ß-hydroxysteroid dehydrogenase in rat tissues: organ specific utilisation of NAD or NADP. *J Endocrinol* 1992;132(Suppl):A101.

3. Benediktsson R, Seckl JR, Edwards CRW. Placental 11ß-hydroxysteroid dehydrogenase: Relationship with fetal and placental size at term. *74th Annual Meeting of the Endocrine Society*, San Antonio, Texas 1992:A622

4. Edwards CRW, Walker BR, Benediktsson R, Seckl JR. Apparent mineralocorticoid excess (AME). *Proceedings of the Ninth International Congress of Endocrinology* 1992.

5. Benediktsson R, Burt D, Lindsay RS, Seckl JR, Edwards CRW. Blood pressure and birth weight: is foetal glucocorticoid exposure the missing link? *J Hypertension* 1992;10:1434.

6. Lindsay RS, Benediktsson R, Noble J, Seckl JR, Edwards CRW. Dexamethasone treatment of pregnant rats leads to raised blood pressure in the offspring. *J Hypertension* 1992;10:1431-1432.

7. Benediktsson R, Edwards CRW, Calder AA Seckl, JR. Cortisol metabolism in the perfused human placenta. *J Endocrinol* 1994;140:P6.

8. Phillips GJ, Langley-Evans SC, Benediktsson R, Seckl JR, Edwards CRW, Jackson AA. The role of dietary protein restriction during pregnancy on the activity of placental 11ß-hydroxysteroid dehydrogenase. *P Nutr Soc* 1994 (In press).

9. Benediktsson R, Brennand J, Tibi L, Calder AA, Edwards CRW, Seckl JR. Cord blood osteocalcin and the placental glucocorticoid barrier. *Scot Med J* 1995 (In press).

10. Benediktsson R, Noble J, Calder AA, Edwards CRW, Seckl JR. Human placental glucocorticoid metabolism and fetal growth. *Scot Med J* 1995 (In press).

11. Benediktsson R, Brennand J, Tibi L, Calder AA, Edwards CRW, Seckl JR. Cord blood osteocalcin: a marker of glucocorticoid exposure in utero? *J Endocrinol* 1995; (In press)

12. Benediktsson R, Noble J, Calder AA, Edwards CRW, Seckl JR 11ß-hydroxysteroid dehydrogenase activity in intact dually perfused fresh human placentas predicts birth weight. *J Endocrinol* 1995; (In press)

13. Benediktsson R, Noble J, Calder AA, Edwards CRW, Seckl JR. 11ß-hydroxysteroid dehydrogenase efficiency in the intact placenta and birth weight in humans. *77th Annual Meeting of the Endocrine Society*, Washington DC, 1995:(In press).

1. INTRODUCTION

1.1. EARLY DEVELOPMENT AND DISEASE

"Lengi býr að fyrstu gerð". This Icelandic proverb is of obscure origin, but was probably first recorded and published in a collection of Icelandic sayings by Guðmundur Jónsson in 1830 [Jónsson 1830]. Its meaning is that early events give a lasting impression. Thus for a long time, the general public appears to have been aware of the importance of early development. This is not unique to Iceland, although an exactly equivalent saying may not exist, and several examples of the same general theme can be found in other cultures' literature. It is only in recent years however, that this has attracted the attention of scientists, who have generated solid data to support this concept. This thesis strives to support this notion, by suggesting a mechanism to explain the phenomenon of prenatal programming of cardiovascular diseases, specifically hypertension.

1.1.1. Epidemiology

In recent years there has been a dramatic shift in our appraisal of the origins of common adult diseases, most notably ischaemic heart disease. These observations have shifted the emphasis from adult "life-style" risk factors (like smoking, exercise and diet) to indicate the importance of early life events. This started when epidemiologists noticed the striking geographical differences in death rates for chronic bronchitis, stroke and ischaemic heart disease. Most of these studies have been conducted in this country by Professor Barker at the MRC Epidemiology Unit in Southampton [Barker 1992a], but similar data has also been gathered in other populations, for example in Norway [Forsdahl 1977] and USA [Buck & Simpson 1982].

The curious paradox apparent in the British geographical data for ischaemic heart disease was that although most common in poorer areas and in lower income groups, the steep increase in incidence during this century had taken place in the most affluent areas [Barker & Osmond 1986; Barker 1988]. These observations suggested that two separate influences were involved; current risk factors and the events of the past [Barker 1988]. In search of past determinants, past neonatal (birth - 28 days), postneonatal (28 days - 1 year) and maternal mortality rates in defined areas of England and Wales were related to current rates of respiratory and cardiac diseases in the same areas.

Using these data it was found that chronic bronchitis could be predicted by past postneonatal mortality, but ischaemic heart disease and stroke correlated better with past neonatal mortality and past maternal mortality [Barker & Osmond 1987; Barker & Osmond 1986], both being indicators of adverse influences at the time of conception and during pregnancy. The data have been criticised for not taking into account the fact that those areas in Britain which were most deprived 50 years ago are also the most deprived areas today [Ben-Shlomo & Smith 1991]. In fact the correlations mentioned above are attenuated if one adjusts for current deprivation and socio-economic status [Ben-Shlomo & Smith 1991]. The level of attenuation partly depends on how one looks at deprivation and socio-economic status, and most of Professor Barker's studies show correlations to be independent of social class (using their definitions). A further study found that in spite of migration, the risk of dying from stroke or ischaemic heart disease could be predicted by the place of birth, the lowest risk being confined to the areas with the lowest infant mortality rates [Osmond et al., 1990], again supporting the view that there are probably two factors to be taken into account, the present and the past.

These were fascinating results which prompted further exploration, this time looking for better and more specific measures of the early life environment and their associations with subsequent disease. Longitudinal data (as opposed to cross sectional studies on populations at two different times) were then obtained, mainly

from two areas in England where unusually detailed obstetrical records were still (luckily) kept, namely Hertfordshire and Preston. This therefore allowed different morbidity parameters currently observed in these populations to be associated with specific indices of development in the same individuals over half a century before. These data were obtained in sizeable cohorts of over 15000 people. Ischaemic heart disease was found to correlate with birth weight (an indicator of foetal development), but better with weight at one year [Barker et al., 1989b], which itself does not correlate well with birth weight [Tanner et al., 1956]. It therefore seemed possible to detach two important early factors, the *in-utero* environment as assessed by birth weight and neonatal influences, estimated by weight at one year.

Looking at the risk factors for ischaemic heart disease, specific patterns could be found. Adult cholesterol metabolism seemed to be partly programmed during infancy [Fall et al., 1992], a notion supported by animal studies [Mott 1986]. Low weight at one year predicted adult elevated fibrinogen concentrations [Barker et al., 1992b], elevated apolipoprotein-B concentrations [Fall et al., 1992], impaired glucose tolerance [Hales et al., 1991] and increased waist-hip ratio [Law et al., 1992], all well recognised risk factors for ischaemic heart disease. Birth weight was also able to predict raised waist-hip ratios [Law et al., 1992].

For blood pressure, a major risk factor for ischaemic heart disease and stroke, the more important determinant was birth weight, implying that very early life events might "programme" blood pressure levels *in-utero* [Barker et al., 1990]. Thus mean systolic blood pressure rose by 11 mmHg as birth weight fell from $>$ 7.5 lb to $<$ 5.5 lb. A very striking observation was that blood pressure also seemed to be linked to placental weight, mean systolic blood pressure rising by 15 mmHg as placental weight rose from $<$ 1 lb to $>$ 1.5 lb. The strongest predictor of blood pressure 50 years from birth was the combination of the two, those attaining the highest adult systolic blood pressures having the lowest birth weight with the largest placentas [Barker et al., 1990]. These data have been interpreted as

indicating different timing and/or magnitude of the growth retarding influence [Barker et al., 1993a] (see later).

It even seemed that it was possible to subgroup the babies further on the basis of placental weights. In the group with smallest placentas, adult hypertension was found in those who at birth had a long thin body and low ponderal index ($^{weight}/_{length^3}$) with reduced head circumference. On the other hand, among those with large placentas, hypertension developed in those who were short in relation to their head size [Barker et al., 1992a]. Grouping them by body measurements only, those with small head circumference and low ponderal index tend to develop Syndrome X (the combination of insulin resistance, hypertension, non-insulin dependent diabetes, and dyslipidaemia) [Barker et al., 1993b] and those who are short in relation to their head size tend to develop the combination of hypertension and high plasma fibrinogen [Barker et al., 1992b].

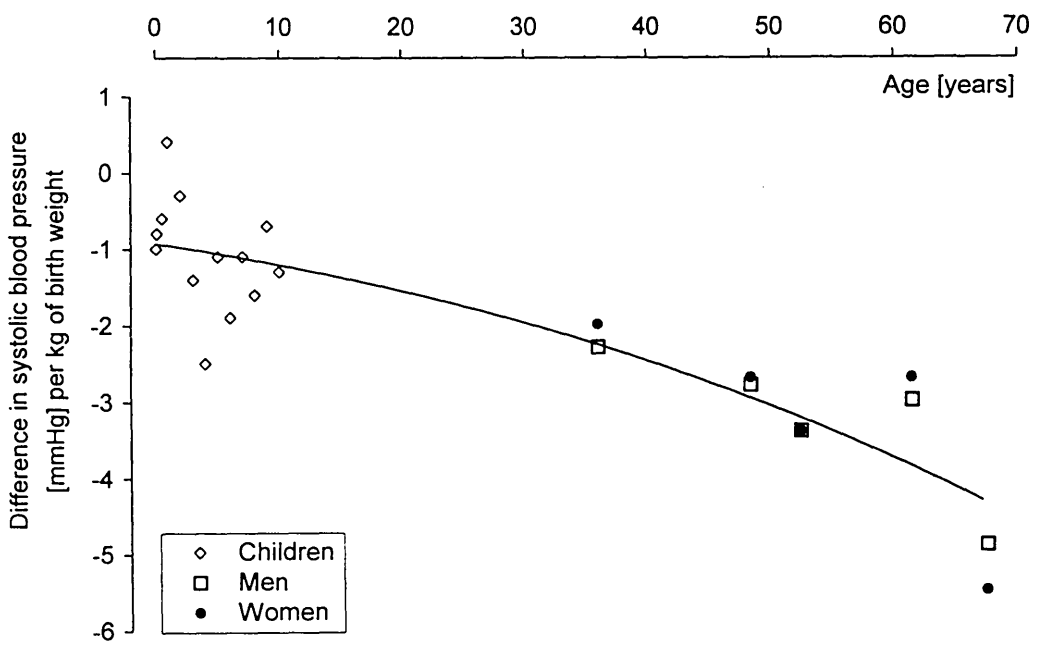


Figure 1.1 Imprinting of Hypertension *In-Utero* and its Self Amplification

The figure shows mean difference (fall in all but one study) in systolic blood pressure per kg increase in birth weight (vertical axis) in groups of people at different current ages (horizontal axis). Adapted from Law *et al.* Brit. Med. J. 1993;306:24-7.

The data for blood pressure have now been confirmed for various populations all over the world, such that we now know that low birth weight predicts elevated blood pressure at all ages [Cater & Gill 1984; Law et al., 1991; Barker et al., 1989a; Seidman et al., 1991; Gennser et al., 1988; Barker et al., 1990]. Important notions to grasp are, that this is not only observed at the extremes of birth- and placental weight distributions but is continuous throughout the normal range [Barker et al., 1990], and the correlations observed occur independently of social class [Barker et al., 1990], adult smoking and obesity [Barker et al., 1989a].

Four of these studies have recently been summarised (Figure 1.1) by Law *et al.* [Law et al., 1993], demonstrating that the effect is observed already in infancy. Indeed, the well recognised "tracking" of blood pressure from infancy to adulthood clearly implicates early events in the determination of blood pressure throughout life [Lever & Harrap 1992]. As can be seen from Figure 1.1, the effect of birth weight on attained systolic blood pressure becomes greater, the older the population studied is. This is could be due to the now well recognised phenomenon of persistence of hypertension and its self-amplification upon removal of the primary cause [Lever & Harrap 1992].

Although the above discussed correlations strongly implicate *in-utero* development in determination of later blood pressure, it is important to appreciate that there are also several other important "current life-style" factors which influence each individuals blood pressure. Growth and current body weight are examples. Thus during teenage years when individuals grow fastest and attain sexual maturation, the relationship between birth weight and blood pressure is weakest [Seidman et al., 1991], and the tracking of blood pressure becomes temporarily perturbed [Lever & Harrap 1992].

1.1.2. Determinants of Birth Weight

Given the persuasive epidemiological observations the key question has to be: What determines birth weight?

There is a strong genetic influence on offspring growth, manifest in the strong correlation of offspring adult height with parental height. Birth weight is not strongly correlated with weight at one year, which however correlates closely with subsequent weight [Tanner et al., 1956]. This must mean that *in-utero*, other local factors are important in determining growth. This is supported by the observation that birth weight is much better predicted by maternal size than paternal size; for example in crosses between large horses and small ponies, where the size of the offspring is directly proportional to maternal size but not intermediate [Hammond 1944]. This notion is also supported by human studies showing that birthweight is influenced by maternal height [Butler & Alberman 1969]. Studies on chimaeric mice [Surani et al., 1990] also support this idea that there are strong local maternal factors capable of modulating foetal growth. In this model, the size of the foetus depends on the relative proportions of maternally vs. paternally derived cells. Thus intrauterine growth is proportionally restrained by up to 50% by the maternal cells and by increasing the proportion of paternal cells the size of the conceptus can be increased.

A popular explanation for the observed correlations between size at birth and adult disease has been that maternal malnutrition is to blame [Barker et al., 1993a]. In support of this, data were produced concerning the Dutch famine during World War II [Stein et al., 1975], where the children were born small and had high risk of subsequent obesity [Ravelli et al., 1976]. There are a number of other points taken in support of the nutrition hypothesis, recently summarised by Barker [Barker et al., 1993a]. A further supportive example in humans was that maternal anaemia and iron deficiency results in the same pattern of increased placental and reduced birth weights [Godfrey et al., 1991] (as does experimental blood loss in

the rat [Alexander 1978]) that predicts higher blood pressure levels. But what is the precise mechanism of malnutrition? An advocated basic mechanism has been alteration of growth factor levels in the foetus; suppression of insulin-like growth factor-1 (IGF-1) is observed in foetal sheep where the mother is subjected to malnutrition, an effect which is restored by glucose infusion but not by amino acid infusion [Oliver et al., 1993].

Could this theory explain why the correlations of birth weight and blood pressure are observed within the normal range of birth weights? This possibility is rather mitigated against by animal studies showing that there must indeed be very severe restriction in maternal nutrition before any effect is noted on birth weight. Thus sheep with singleton pregnancies fed restricted diet during the last 60 days of pregnancy resulting in only 0.5 kg weight gain (fully fed controls gained 17.7 kg during the same period) had lambs of similar birth weights [Hammond 1944]. Findings in cattle are similar [Eckles 1919] and in rats, only diet containing $\leq 6\%$ protein (normal laboratory chow is 20 - 22%) has any detrimental effects on birth weight [Langley & Jackson 1994].

Furthermore, in London during the war of 1939 - 1945 (when some of the Preston cohort [Barker et al., 1990] were *in-utero*) there was no evidence of an effect of the wartime diet on birth weight or the frequency of prematurity [Huggett 1944], and wartime maternal supplementation with minerals and vitamins did not affect birth weight [Anonymous 1942]. Indeed, the Dutch famine of World War II may be an extreme exception or may be related to non-dietary stress (see later). Another problem is why the correlations hold at each level of social class, irrespective of whether it is determined at birth by fathers occupation or at the time of blood pressure measurement [Barker et al., 1993b]. Further, although relatives may share the same environment and/or nutritional patterns, why is intrauterine growth retardation (IUGR) more common in offspring of relatives of mothers that

have given birth to growth retarded children [Morton 1955] and in offspring of those mothers who themselves suffered IUGR [Ounsted & Ounsted 1966]?

The type of growth retardation is also important; symmetrical growth retardation is caused by adverse influences early in pregnancy while influences acting only in late pregnancy (like placental insufficiency) result in asymmetric body proportions with brain sparing [Brar & Rutherford 1988; Kramer et al., 1990; Urrusti et al., 1972; Villar & Belizan 1982; Wigglesworth 1989; Chiswick 1985]. One of Barker's studies [Law et al., 1991] could identify three different patterns of growth abnormalities predicting high blood pressure, each consistent with influences operating during different periods of intrauterine life (although these data can be criticised for the "post-hoc" determination of associations from subpopulations). One tends to think of the mother and foetus as unity, and thus it at first seems inconceivable that human malnutrition should only be present during specific periods of pregnancy although examples of separate involvement are in fact available, such as hyperemesis gravidarum and late placental insufficiency.

However, symmetric growth retardation in animals can only be produced by severe protein-calorie malnutrition [Straus et al., 1991], when no brain sparing is observed [Brar & Rutherford 1988], in contrast to the observations in the Preston study [Barker et al., 1990]. Those suffering from this symmetric type of IUGR exhibit poor catch-up growth in infancy [Brar & Rutherford 1988], while humans with the highest adult blood pressures are those who have grown fastest during childhood [Lever & Harrap 1992].

In summary, there are problems with the malnutrition hypothesis and to explain the associations between low birth weight and subsequent elevated blood pressure (and other diseases), one has therefore to consider the explanation as yet unknown. Even if one can get aetiological clues from severe IUGR, there might be different factors acting to produce the patterns observed within the normal ranges of birth and placental weights. There could even be multiple factors capable of inducing

offspring hypertension, each acting through different (or common) mechanisms and/or at different times during pregnancy. Thus there are now three papers from separate groups reporting *in-utero* programming of hypertension using different approaches; severe maternal protein-calorie malnutrition [Langley & Jackson 1994], moderate maternal glucocorticoid excess [Benediktsson et al., 1993], and a much older paper where numerous (less well characterised) different pharmacological maternal manipulations could induce offspring hypertension [Grollman & Grollman 1962].

1.1.3. Programming

What could be the basic mechanism by which the factor(s) responsible for the above observations act? Several studies have shown that there is perinatal plasticity of hormone receptors and enzymes. Csaba [Csaba 1980] introduced the term "hormonal imprinting" (here called programming) to describe the phenomenon of lasting changes in receptor-ligand interaction, induced by brief perinatal manipulations of the relevant system. In one of his studies he reported lasting amplification of insulin receptor responses after a single neonatal insulin dose [Csaba et al., 1979]. At three months of age the treated animals however only differed in fasting glucose levels (controls higher) and no difference was observed in the response of glucose to insulin injection. Similarly he reported lasting amplification of vasopressin responses in rat aortic strips following a single neonatal injection of vasopressin when compared to controls which received saline only [Csaba et al., 1980]. However, the differences were small and proved not to be specific as the responses of the aortic strips to noradrenaline were also increased [Csaba et al., 1980].

The permanent effects of steroid hormones given during the perinatal period are better established. Brief neonatal exposure to sex steroids leads to permanent organisational changes in neuroanatomy, neuronal chemistry and behaviour, which persists throughout adult life [Arai & Gorski 1968]. Furthermore, there is strong

evidence that steroids cause irreversible programming at birth of both microsomal and soluble rat liver enzymes [Gustafsson & Stenberg 1974b; Gustafsson & Stenberg 1974a]; a single neonatal testosterone treatment permanently induces liver 5ß-reductase, whereas 5α-reductase is suppressed throughout life.

The low birth/placental weight ratio predicting hypertension is found in maternal anaemia [Godfrey et al., 1991] as stated above, but is also found in people living at high altitudes [Krüger & Arias-Stella 1970], where the foetus similarly is chronically hypoxic. The disproportionate placental enlargement has in both cases been thought to be compensatory or secondary. Are there any other situations where this peculiar pattern of birth and placental weights has been observed?

Smoking results in a proportional reduction in birthweight and length [Miller & Hassanein 1974], consistent with an influence acting throughout pregnancy (see discussion above in relation to Professor Barker's findings). The effect of smoking on placental weight is somewhat controversial. One study found no effect of maternal smoking [Spira et al., 1975], while another found smoking reduced placental weight as well as birth weight, but disproportionately, increasing the ratio of placental to birth weight [Godfrey et al., 1991]. We do not know whether maternal smoking leads to offspring hypertension. The effect on offspring blood pressure of alcohol administration to pregnant mothers is similarly not known, but chronic alcohol administration to pregnant rats leads to low foetal weight and high placental weight [Sanchis et al., 1986].

Rats made diabetic with streptozotocin give birth to low weight offspring that also have high placental weights [Sybulski & Maughan 1971; Robinson et al., 1988; Canavan & Goldspink 1988]. These effects are only partially reversed by insulin treatment, and interestingly these animals have markedly raised maternal glucocorticoid levels [Heller et al., 1988] and foetal plasma amino acid levels are significantly lower than in controls, while maternal amino acid levels are normal [Martín et al., 1990]. By contrast, alloxan-induced diabetic rats have normal

maternal glucocorticoid levels and do not exhibit reduced birth or increased placental weights [Sybulski & Maughan 1971].

It is now clear that glucocorticoids retard foetal growth both in humans [Reinisch et al., 1978; Katz et al., 1990] and animals [Benediktsson et al., 1993]. For placental weight, glucocorticoids may increase or reduce placental weight [Gunberg 1957], probably depending on dose and/or timing of exposure. Of course, glucocorticoids directly elevate blood pressure in adults (Cushing's syndrome for example) and even *in-utero*, at least in foetal sheep [Tangalakis et al., 1992].

There are several mechanisms by which glucocorticoids may affect blood pressure. Direct effects on vasculature *in-utero* could be mediated via enhanced vascular responses to vasoactive agents like noradrenaline [Russo et al., 1990] and angiotensin-II [Tangalakis et al., 1992] or by regulation of prostacyclin synthesis in blood vessels [Danon & Prajgrod 1991] (for a review of direct regulatory mechanisms, see Walker & Williams 1992). Indirect effects (programming) affecting blood pressure could be the result of glucocorticoids altering *in-utero* brain development [Mayer 1985]. Neonatal stress for example permanently programmes patterns of hypothalamic-pituitary-adrenal responses, effects mediated largely through altered expression of glucocorticoid receptor genes in the brain [LaRocque et al., 1992; Meaney et al., 1993]. Development of several organs other than brain is also altered by prenatal glucocorticoids, for example heart and kidney [Slotkin et al., 1992b; Bian et al., 1993], both of which could be implicated in blood pressure control. Central noradrenergic activity is also permanently altered by brief prenatal glucocorticoid exposure [Slotkin et al., 1992a], as is peripheral sympathetic innervation, adrenergic receptor expression and adenylate cyclase function [Bian et al., 1992; Bian et al., 1993]. For a review of organs/enzymes affected, see Ballard 1979.

Recently, we demonstrated that treatment of pregnant rats with dexamethasone, in a modest dose which reduced average birth weight by 14% and did not alter litter size or gestation length, caused elevated blood pressures in the adult offspring, more than 5 months after exposure to exogenous glucocorticoid [Benediktsson et al., 1993]. Although the precise mechanism in this example is not known, with reference to the above discussion, several mechanisms can be invoked.

As mentioned above, there are indications that malnutrition might affect the expression of growth factors, providing a basic mechanism by which malnutrition might act since there are now strong indications that foetal growth is to a large extent controlled by IGF-1 and IGF-2 in harmony with their respective binding proteins (IGFBP-1 and IGFBP-2) and at least two different receptors. There is now a body of evidence showing that glucocorticoids modulate the expression of IGF-2, the type-1 IGF receptor and the binding proteins 1 and 2 [Luo et al., 1990]. All are altered in foetal liver following dexamethasone treatment of pregnant rats [Price et al., 1992], and direct infusion of cortisol into foetal sheep markedly reduces hepatic IGF-2 mRNA expression [Li et al., 1993]. Interestingly, the streptozotocin diabetic rat, which as stated before has foetal growth retardation, high placental weight and elevated maternal glucocorticoid levels, even has raised foetal IGFBP-1 and IGFBP-2 expression [Ooi et al., 1990; Heller et al., 1988].

So are glucocorticoid hormones likely candidates in man? Could glucocorticoids be the basic mechanism explaining the maternal malnutrition hypothesis? Chromosomally normal human foetuses suffering IUGR have elevated cortisol levels as well as elevated corticotropin-releasing hormone [Goland et al., 1993] and although data from IUGR cases has to be interpreted with care, this can be taken as supporting evidence. What precisely are glucocorticoid hormones and what controls their access to the growing foetus?

1.2. HORMONES

The term hormone is derived from Greek, meaning "to excite or stir up". Hormones are substances produced by a cell/tissue. Following secretion, hormones affect their target tissue(s)/cell(s). Hormones affecting the cell which secreted the hormone, are termed Autocrine, while if the target is another cell locally the hormone is termed Paracrine. Endocrine hormones on the other hand are secreted by one tissue and have their effects on another (distant) tissue, classically carried there by the bloodstream.

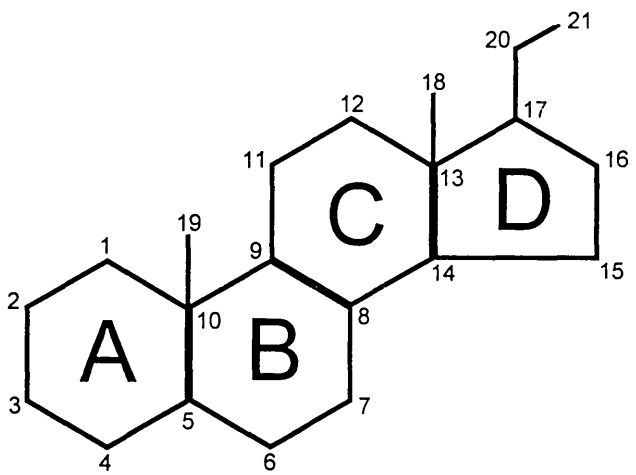


Figure 1.2 Basic Structure of Steroids.

The basic four ring structure (cyclopentanoperhydrophenanthrene) of all steroid hormones. The rings themselves are named A-D while each carbon atom has a number. On the basis of carbon atoms, classes of hormones can be identified. C18: oestrogens, C19: androgens, C21: gestagens, gluco- and mineralocorticoids. Adapted from [Orth et al., 1992a].

Biochemically one can classify endocrine hormones as peptides (e.g. insulin), amines (e.g. thyroxine) or steroids (e.g. cortisol). Effects are mediated following binding of the hormone to specific molecules (Receptors) on the target cell surface or within the cell. This either results in an effect or forms (or sets free) an intracellular molecule (Second Messenger) which brings about the effect. Although

certain generalisations about hormones' synthesis, secretion and control are possible, the subject is becoming more complex. Regulation of action generally occurs at several levels: synthesis, secretion, carriage in the circulation, distribution, clearance, tissue uptake and/or receptor binding and at post-receptor level.

1.3. CORTICOSTEROID HORMONES

Steroid hormones are variably lipid soluble, having a common derivation from the cyclical alcohol cholesterol, which itself is synthesised from acetyl coenzyme A (acetyl-CoA). Examples of steroid hormones are the male and female sex hormones, and the adrenal corticosteroids (mineralo- and glucocorticoids). The basic structure of steroid hormones is shown in Figure 1.2. The adrenal cortex, ovaries and testes are embryologically related and all have the capacity for synthesis of steroid hormones. A limited account of steroidogenesis in the adrenal cortex follows below.

1.3.1. Synthesis

The human adrenal gland is recognisable by the second month of gestation and is functionally active from early in pregnancy [Murphy 1973]. At mid-gestation it is bigger than the kidney, being composed of an outer cortex, and inner medulla which is formed by invasion of the gland by embryologically unrelated tissue of neural crest origin. At term, two zones can be distinguished in the cortex, the bulk provided by the inner foetal zone which rapidly degenerates following birth. The outer definitive cortical zone by birth has two distinct subdivisions: outer zona

glomerulosa and inner zona fasciculata. It is only during the first year of life that the innermost zona reticularis develops.

Microscopically the distinction between these three areas is not very sharp, and knowledge about the factors regulating growth of the adrenal cortex is incomplete. Functionally however, this zonation makes sense, the foetal zone mainly producing dehydroepiandrosterone sulphate, while mineralocorticoid synthesis takes place in the glomerulosa and glucocorticoid synthesis in fasciculata/reticularis. The adrenal cortex is capable of *de-novo* cholesterol synthesis, although about 80% of the requirements for steroid biosynthesis are provided by the plasma pool of cholesterol. The principal members of the three major classes of steroids (glucocorticoids, mineralocorticoids and androgens) produced by the adrenal cortex in humans, are cortisol, aldosterone and dehydroepiandrosterone respectively. The biosynthetic pathways are shown in Figure 1.3.

The physiological glucocorticoid in humans is cortisol, a 21 carbon (C21) steroid formed in five steps from cholesterol in the zona fasciculata/reticularis. Four of the enzymes required are cytochrome P-450 enzymes. The physiological glucocorticoid in rats is corticosterone as these animals lack the enzyme 17α-hydroxylase. As indicated in Figure 1.3, the first step is cleavage of the side chain of cholesterol between carbons 20 and 22. This is effected by cholesterol desmolase (P-450 SCC), which is regulated by ACTH [Waterman & Simpson 1989]. This enzyme is mitochondrial and the rate limiting step in the biosynthetic pathway. This side chain cleavage of cholesterol produces pregnenolone which then enters the cytoplasm where it is converted to progesterone by 3ß-hydroxysteroid dehydrogenase (the only non P-450 enzyme). 17α-hydroxylase can convert both pregnenolone and progesterone to their respective 17α-hydroxy

varieties. 17α-hydroxylase has 17,20-lyase activity which is responsible for conversion of 17-hydroxypregnenolone to the principal adrenal androgen, dehydroepiandrosterone.

To form cortisol, 17-hydroxyprogesterone first undergoes 21-hydroxylation by a P-450 enzyme in the smooth endoplasmic reticulum thus generating 11-deoxycortisol. The last step is then 11ß-hydroxylation by a fasciculata specific isoform [White et al., 1994] of 11ß-hydroxylase (correct nomenclature is now CYP11B1 [Nelson et al., 1993]). Going down the left pathway (escaping 17α-hydroxylation) in Figure 1.3, progesterone is acted upon by 21-hydroxylase giving rise to 11-deoxycorticosterone. 11ß-hydroxylation then occurs by CYP11B1 thus generating small amounts of corticosterone in the zona fasciculata.

In the zona glomerulosa, which functions to generate mineralocorticoids, mainly under the control of angiotensin-II and potassium [Quinn & Williams 1988], pregnenolone is converted into progesterone which in turn is 21-hydroxylated (Figure 1.3) to give 11-deoxycorticosterone. Corticosterone is then generated by the second isoform of 11ß-hydroxylase, CYP11B2, which is a product of a gene different from the gene for CYP11B1 [Mornet et al., 1989]. CYP11B2 does not stop at 11ß-hydroxylation like its counterpart in zona fascicularis, but goes on to generate aldosterone in two successive steps, 18-hydroxylation and 18-oxidation, the substrate probably never leaving the enzyme [White et al., 1994].

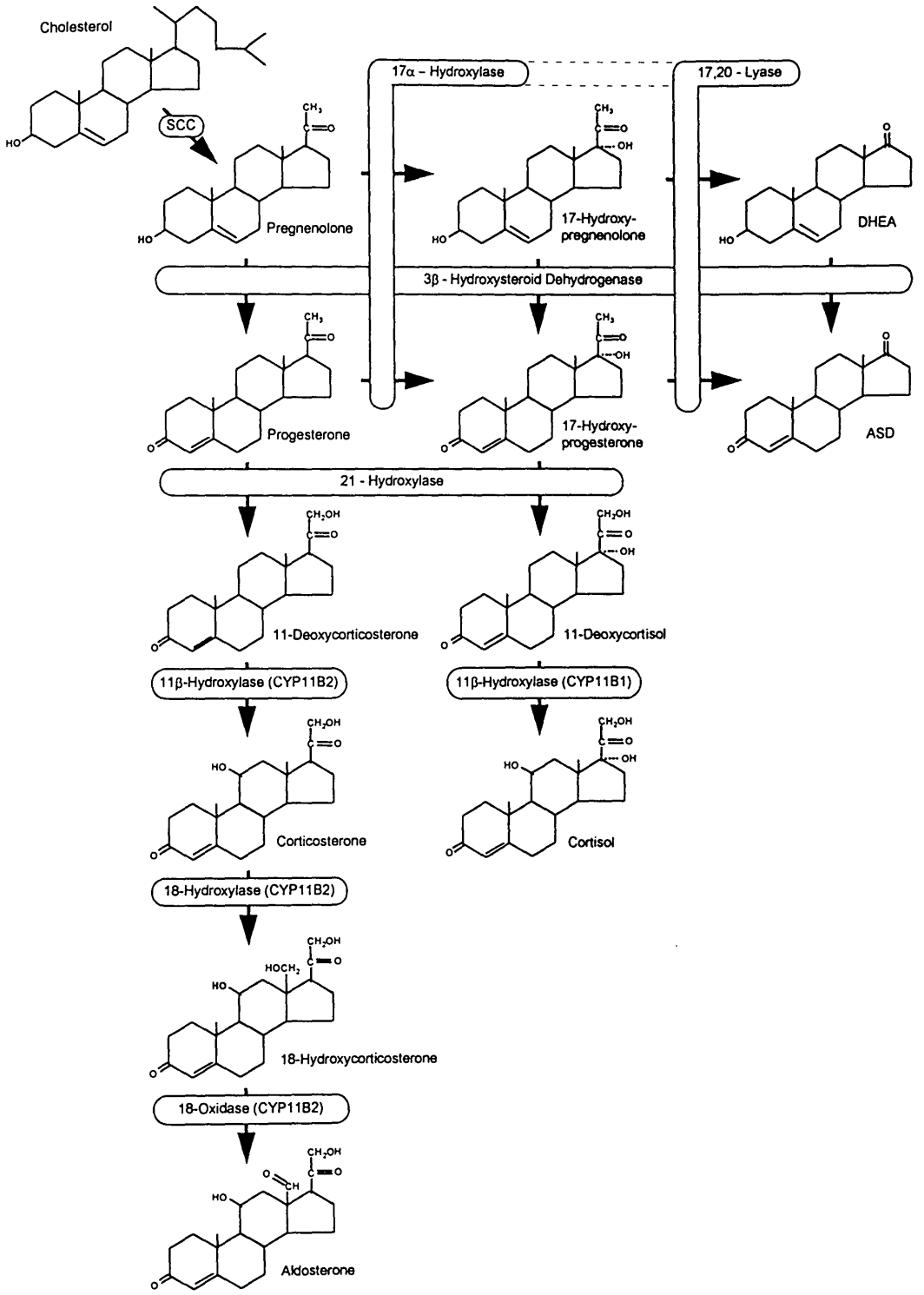


Figure 1.3 Biosynthesis of Adrenocortical Steroids

The figure is modified from several sources (see text for discussion). 17α-hydroxylase also has 17,20-lyase activity. CYP11B1 and CYP11B2 are products of separate genes, both can 11ß-hydroxylase. The last three steps of aldosterone synthesis are carried out by a single enzyme (CYP11B2). SCC: Side Chain Cleavage effected by cholesterol desmolase; DHEA: Dehydroepiandrosterone; ASD: Androstenedione.

1.3.2. Regulation

The discussion will be limited to glucocorticoids. The integration of glucocorticoid homeostasis is complex and involves various regions in the brain, the pituitary gland, the adrenal cortex and target organs as well as plasma proteins. An overview is given in Figure 1.4. The main stimulus to glucocorticoid secretion from the adrenal cortex is ACTH [Waterman & Simpson 1989; White et al., 1994]. Corticotropin (ACTH - AdrenoCorticoTropic Hormone) is a 39 amino acid peptide secreted from the anterior pituitary but the first 18 NH_2-terminal amino acids are the biologically active part of the molecule. Its synthesis involves translation and processing of a 241 amino acid precursor peptide called proopiomelanocortin (POMC) [Eipper & Mains 1980].

Acutely, ACTH increases cortisol synthesis (adrenal cortisol storage is minimal) by inducing side chain cleavage (cholesterol desmolase), converting cholesterol into pregnenolone. ACTH acts through a G protein-coupled receptor [Mountjoy et al., 1992], which increases cAMP levels, resulting acutely in increased activity of cholesterol desmolase but also (chronic effects) affects transcription of genes encoding the enzymes in the biosynthetic pathway, thereby increasing adrenal weight [John et al., 1986; Gill 1972].

Multiple hormonal factors affect ACTH secretion [Antoni 1986; Antoni 1993], but the most important positive stimulus is a hypothalamic 41 amino acid peptide called CRF (Corticotropin Releasing Factor), which acts through adenylate cyclase/cAMP. CRF is secreted under multiple influences from other areas in the brain, including the limbic system. Its actions on ACTH secretion are potentiated by Vasopressin (AVP) which itself stimulates ACTH secretion directly. In

addition, catecholamines, angiotensin-II, serotonin, oxytocin, atrial natriuretic factor, and several other factors have been implicated [Antoni 1986; Antoni 1993].

Negative feedback is an important feature of this homeostatic system and while cortisol itself does not appear to exert negative feedback on its biosynthetic pathway in the adrenal it does so on ACTH secretion and POMC transcription [Lundbland & Roberts 1988]. At the level of the hypothalamus (CRF and AVP), cortisol and perhaps ACTH exert negative feedback. Steroid receptors are found in many areas of the brain, for example the hippocampus which is also part of the feedback system.

The plasma levels of both cortisol and ACTH exhibit a diurnal rhythm [Krieger et al., 1971; Weitzman et al., 1971; Veldhuis et al., 1990], the mechanism for which is not clear. ACTH secretion is pulsatile throughout the 24 h but the pulse amplitude increases a few hours after onset of sleep in humans. Cortisol levels peak a few hours later, therefore usually in the early morning, declining to a nadir at midnight. Although there is probably an endogenous pacemaker in the hypothalamus (the suprachiasmatic nucleus) which controls the rhythmicity of the above system, environmental stimuli play an important role. The system, which collectively is called the Hypothalamic-Pituitary-Adrenal Axis, is affected by multiple physical and psychological stresses (feeding, trauma, hypoglycaemia, smoking, exercise, etc.).

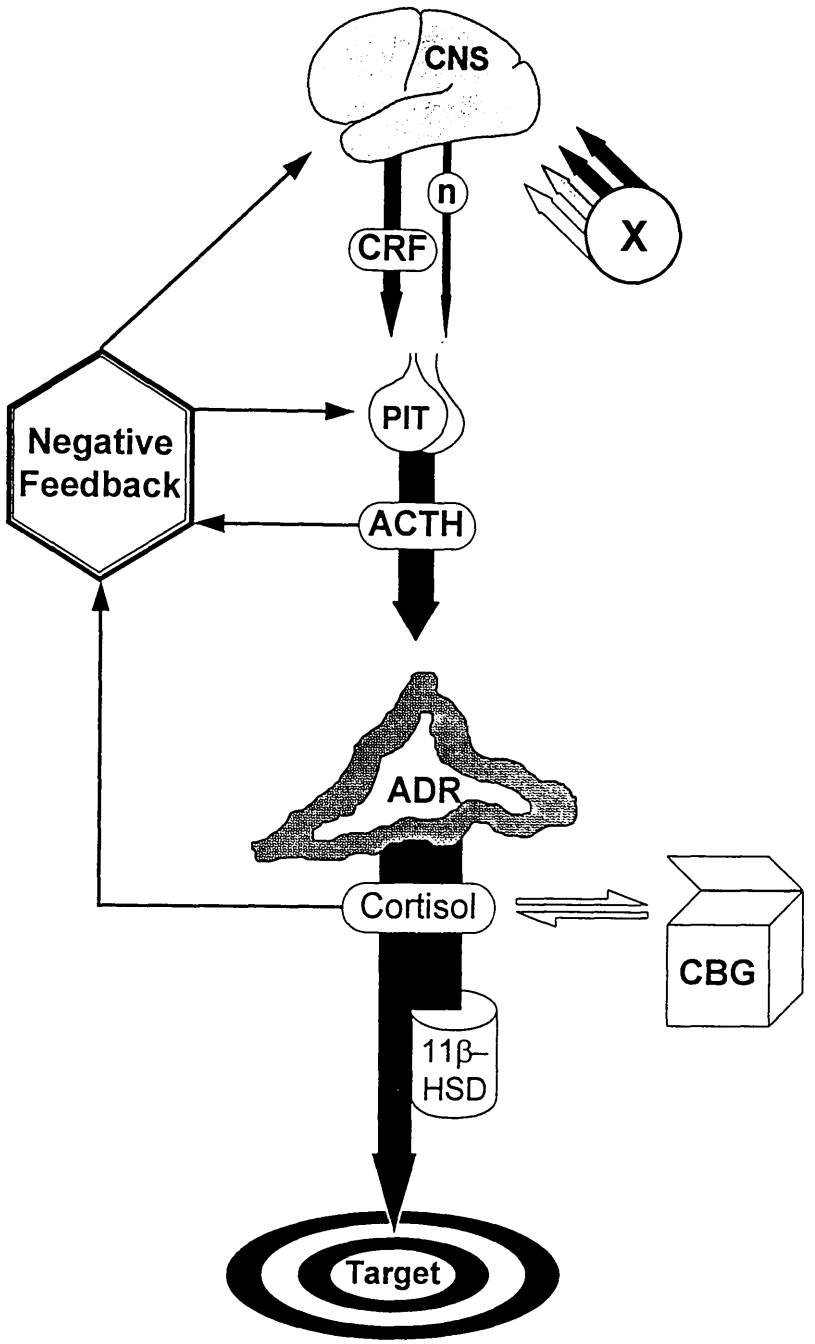


Figure 1.4 A Schematic Outline of the Hypothalamic-Pituitary-Adrenal Axis

CRF_{41}, vasopressin and other factors stimulate ACTH secretion which in turn stimulates cortisol synthesis by the adrenal cortex. Plasma levels of free cortisol are balanced mainly by CBG which is synthesised mainly in the liver and target tissue metabolism is effected by 11ß-HSD. Negative feedback as indicated; environmental stimuli are important as is neural activity within the CNS. Refer to text for fuller description. PIT: pituitary gland. CRF: corticotropin releasing factor. n: multiple hormonal stimuli including vasopressin. X: multiple environmental physical and psychological stimuli. 11ß-HSD: Target tissue metabolism by 11ß-hydroxysteroid dehydrogenase. CNS: central nervous system. ADR: adrenal gland. ACTH: adrenocorticotropin hormone. CBG: corticosteroid binding globulin.

1.3.3. Metabolism

Biological activity of cortisol is believed to relate to the plasma fraction which remains free in circulation. This is normally in the low nM range in humans, about 3 - 4% of plasma total cortisol or corticosterone. Most of circulating glucocorticoid is attached to plasma binding proteins, rendering the steroid biologically inactive, but protecting the molecule from degradation. The binding proteins are corticosteroid binding globulin (CBG) with which about 90% of cortisol (ca. 80% of corticosterone) is associated, albumin and a small proportion is associated with testosterone binding globulin (TeBG or Sex Hormone Binding Globulin: SHBG) [Dunn et al., 1981]. Albumin has greater capacity than CBG for cortisol binding due to higher concentration, although the affinity is much lower. During diurnal peaks and at times of stress, albumin will thus dampen fluctuations in free cortisol levels as CBG is flooded due to its low capacity (although high affinity) [Orth et al., 1992e].

Anatomically, the metabolism of cortisol can conveniently be divided into hepatic and extrahepatic, the latter mainly effected by 11ß-hydroxysteroid dehydrogenase (see Section 1.4 page 40). The biochemical pathways in the liver involve oxidation, reduction, hydroxylation and conjugation [Orth et al., 1992b]. The structural features important for high affinity binding to the glucocorticoid receptor are: Δ^4 configuration, 11ß- and 21-hydroxy group as well as a 3- and 20-keto group [Orth et al., 1992d]; modification at these points is therefore most important. Accordingly, A-ring reduction is a major liver pathway, shown as steps A_1 and A_2 in Figure 1.5, involving reduction of both the 3-keto and Δ^4 double bond, generating tetrahydrocortisol. Oxidation of 11ß-hydroxyl group (B) is similarly important and is considered in Section 1.4 page 40. Various ways of inactivating cortisol by modifying the C17 side chain exist. Step (C) indicates complete side chain cleavage while (D) indicates reduction of the 20-keto group. 6ß-hydroxylation (E) is not very important in normal individuals, but in cases of glucocorticoid excess (e.g. Cushing's syndrome) disproportionally high urinary

excretion of 6ß-hydroxycortisol is observed, probably due to saturation of normal pathways [Voccia et al., 1979]. Not depicted in Figure 1.5, is conversion of the CH_2OH configuration of carbon 21 to a COOH configuration which cortisol and its other metabolites can undergo. Finally, the various metabolites can be conjugated to glucuronide to render them more water soluble.

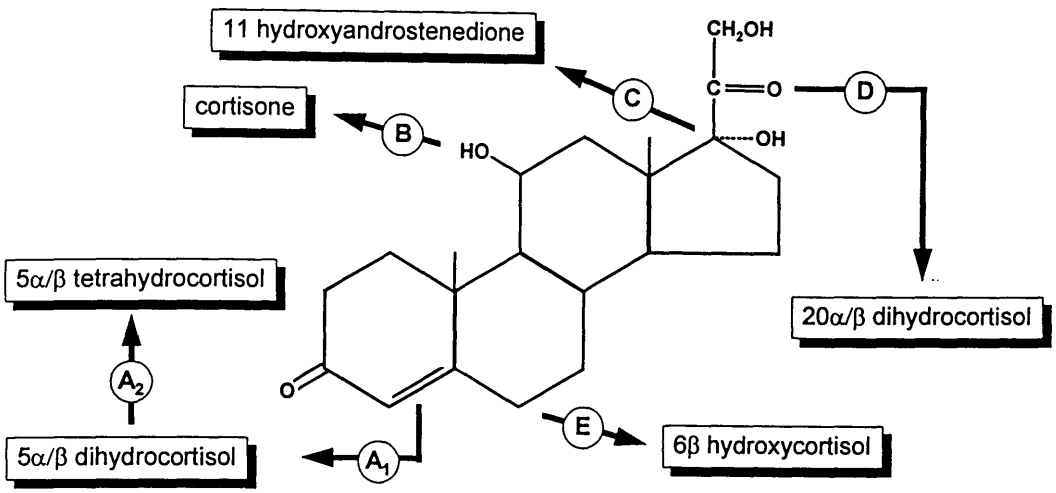


Figure 1.5 Metabolism of Cortisol

The figure shows major pathways of cortisol inactivation. α and ß indicate two possible isomers. See text for full discussion.

1.3.4. Role

Glucocorticoids have myriad roles. Perhaps it is easiest to get an overall view by considering disease states. If present in excess, like as Cushing's Syndrome or following long term treatment with exogenous glucocorticoids, morphology changes. There is redistribution of fat, becoming centripetal; osteoporosis develops and degeneration of muscle and connective tissue is observed; growth is impaired. The two latter factors lead to the clinical observations of bruising, weakness, wasted muscles and thin skin. The patient has hypertension, sexual dysfunction and reduced resistance to infections. Glucose intolerance or diabetes may develop and psychological changes like depression are frequently observed. Glucocorticoid deficiency states (e.g. Addison's disease), are associated with

hypoglycaemia, weight loss, fluid and electrolyte disturbances (inability to excrete a water load).

For fuel homeostasis in more detail; glucocorticoids promote gluconeogenesis and glycogen deposition in the liver by inducing the activity of several specific enzymes (e.g. glucose-6-phosphate, phosphoenol pyruvate carboxykinase) [Exton 1979] as well as by inhibiting the activity of glycogenolytic enzymes (glycogen phosphorylase) [Stalmans & Laloux 1979]. These effects are anabolic, aiming at elevating plasma glucose levels. Peripherally the actions are classified as catabolic, since glucose transport into cells is inhibited, (again keeping plasma glucose high) [Munck 1962], while lipolysis is enhanced (for example sensitivity to catecholamines increased) [Exton 1979; Fain 1979], and amino acids are mobilised from skeletal muscle (substrate for gluconeogenesis). Biochemically this leads to elevated plasma glucose and free fatty acids.

In addition to the above effects on muscle and connective tissue, osteoporosis results from excessive glucocorticoid exposure, due to inhibition of osteoblastic activity [Cheng et al., 1994]. Glucocorticoids also inhibit Ca^{++} absorption from the gut [Lukert & Adams 1976], and by decreasing renal Ca^{++} reabsorption they enhance urinary calcium excretion [Laake 1960].

Glucocorticoids affect immunological function profoundly [Orth et al., 1992c], which is manifest clinically by immunodeficiency and predisposition to infection. Distribution of the cells of the immunological system is modulated and so is their behaviour. Not only do they inhibit proliferation/maturation of T- and B-lymphocytes but also the release of mediators from them. Thus prostaglandin and leukotriene homeostasis is modulated.

Fluid and electrolyte homeostasis is also modulated by glucocorticoids. In the gut, ion transport is directly affected by glucocorticoids and glucocorticoids do have affinity for mineralocorticoid receptors as discussed in Section 1.4 on page 40. It is also well established clinically that glucocorticoid excess (Cushing's) is

associated with hypertension. This effect has been reproduced in animals chronically infused with dexamethasone [Tonolo et al., 1988] and in foetal sheep, cortisol infusion increases blood pressure, possibly via increased angiotensin-II sensitivity [Tangalakis et al., 1992]. The mechanism has yet to be fully explained, but may relate to increased vascular sensitivity to vasoconstrictors via increased adrenergic and/or angiotensin receptor expression, increased 2^{nd} messenger responses (induction of adenylate cyclase) and/or increased substrate production (angiotensinogen, tyrosine hydroxylase induction) (for further discussion, see Section 1.1.3, page 24, and Walker & Williams 1992).

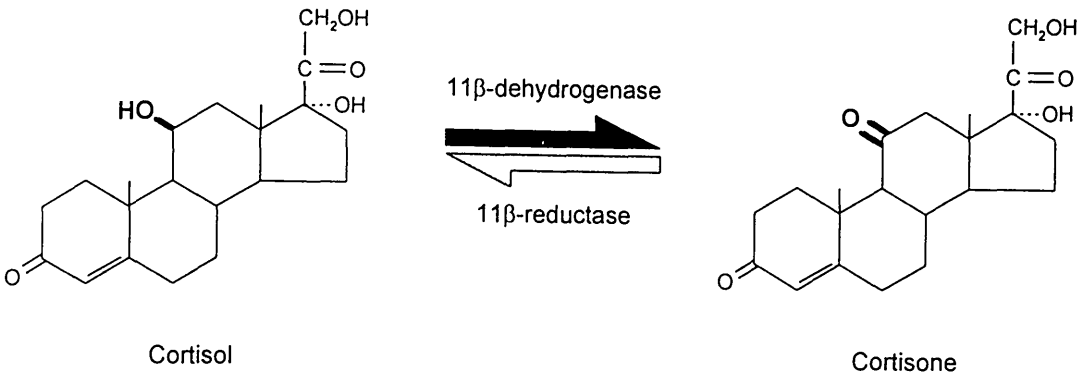
As regards the central nervous system (CNS), glucocorticoids have multiple effects on behaviour, the clinical literature abounding with accounts of psychosis following administrations of synthetic glucocorticoids. In fact over half of patients with Cushing's are thought to have psychological disturbances, typically depression and cognitive/memory dysfunction [Plotz et al., 1952; Jeffcoate et al., 1979; Starkman et al., 1986]. Many of these effects are likely to represent effects via mineralocorticoid and glucocorticoid receptors which are present on neurons, although some of them occur so rapidly that it is unlikely that they are operating via classical steroid receptor-gene interactions [Orth et al., 1992f]. On the other hand there is also now clear evidence for glucocorticoids affecting development of the CNS, and they are directly toxic to neurons causing cell death, largely by increasing neuronal vulnerability to a wide range of other insults by inhibiting glucose uptake and by potentiating intracellular Ca^{++} release [Virgin et al., 1991; Elliot & Sapolsky 1993].

For discussion on glucocorticoids and development see Section 1.1.3.

1.4. 11ß-HYDROXYSTEROID DEHYDROGENASE

11ß-hydroxysteroid dehydrogenase (11ß-HSD) is a widely distributed enzyme catalysing the interconversion of ß-hydroxy and keto groups in position 11 in a number of steroids (Figure 1.6) (for a list of over 30 steroid substrates, see Monder & White 1993). 11ß-HSD activity in the liver is responsible for the biological activity of cortisone, being converted to active cortisol, while in the kidney its function is the opposite, inactivation of cortisol conferring specificity to the mineralocorticoid receptor [Edwards et al., 1988]. It is now clear that at least two isoforms of 11ß-HSD exist, 11ß-HSD-1 [Krozowski et al., 1992; Tannin et al., 1991; Moore et al., 1993] and 11ß-HSD-2 [Albiston et al., 1994; Krozowski 1994; Brown et al., 1994; NarayFejesToth et al., 1994b; Stewart et al., 1994b; Stewart et al., 1994a].

Evidence for the interconversion of steroids with 11-hydroxy and 11-keto groups was available already in the early 1950's [Hechter et al., 1951; Mason 1950]. In 1953 Amelung [Amelung et al., 1953b; Amelung et al., 1953a] described a rat liver enzyme capable of catalysing this conversion. The enzyme was called "11ß-hydroxy dehydrogenase" by Hübener in 1956 [Hübener et al., 1956] but is now termed "11ß-hydroxysteroid dehydrogenase". In 1960, Osinski [Osinski 1960] described the enzyme in the placenta, where it has been thought to protect the foetus from the much higher maternal glucocorticoid levels. It was however not until Edwards and Stewart [Stewart et al., 1988] described a case of the Syndrome of Apparent Mineralocorticoid Excess (AME), that the importance of this enzyme was realised.



Cortisol

Cortisone

Figure 1.6 11ß-Hydroxysteroid Dehydrogenase

The figure shows the reaction catalysed by 11ß-HSD in man. The physiological glucocorticoid in the rat is corticosterone, and 11-dehydrocorticosterone is the cortisone equivalent. These substances differ from their human counterparts only in the lack of an OH group in position 17α. The groups involved are indicated in bold.

1.4.1. Syndrome of Apparent Mineralocorticoid Excess

When presented with a patient suffering hypertension in conjunction with hypokalaemia, aldosterone excess immediately springs to mind. Over the last two decades however, a rare syndrome of hypertension with hypokalaemia and intriguingly, suppressed plasma renin activity and aldosterone has emerged. More than 20 children with this disorder have now been diagnosed, distinguished from Liddle's syndrome by failure of the latter to respond to spironolactone or dexamethasone suppression of the hypothalamic-pituitary-adrenal axis. Other disorders like congenital adrenal hyperplasia and primary glucocorticoid resistance, in contrast to AME have raised 11-deoxycorticosterone (DOC) (they also have abnormal levels of other adrenal steroids). Clinical features in addition to hypertension in children with AME are short stature, failure to thrive, polyuria and polydipsia.

Although in retrospect the first case [Werder et al., 1974] was described in 1974, it was not until New and colleagues described similar cases [New et al., 1977; Ulick et al., 1979] that the term Apparent Mineralocorticoid Excess (AME) was coined

for this disorder. The term "apparent" was appropriate, as an intensive search for a hitherto unknown mineralocorticoid steroid was fruitless and 11-deoxycorticosterone (DOC) levels were not raised. The salient abnormality in this syndrome is a defect in the peripheral metabolism of the physiological glucocorticoid cortisol. Thus urinary 11ß-hydroxy metabolites of cortisol (tetrahydrocortisol: THF and allo-tetrahydrocortisol: allo-THF) were raised in comparison to the 11-oxo metabolites (cortisone-metabolites i.e. tetrahydrocortisone: THE) indicating impaired conversion of the active glucocorticoid cortisol to its inactive counterpart cortisone [Stewart et al., 1988]. The half life of cortisol was thus prolonged, estimated by conversion of 11α^3H-cortisol to unlabelled cortisone and tritiated water, indicating impaired oxidation of the 11-hydroxyl group [Ulick et al., 1979; Stewart et al., 1988]. Cortisol levels in peripheral blood were normal however, maintained by reduced adrenal cortisol secretion rate via altered hypothalamic/pituitary feedback .

These children responded to pituitary-adrenal suppression by administration of the pure glucocorticoid dexamethasone. Treatment with hydrocortisone aggravated the condition leading to the suggestion that cortisol was the mineralocorticoid involved [Oberfield et al., 1983].

Ulick and New [New et al., 1977; Ulick et al., 1979] had described an abnormality of the dehydrogenase component of 11ß-HSD in the children with AME, but the pivotal role of this enzyme in the pathogenesis of the syndrome remained obscure until a 20 year old male presented with severe hypertension, hypokalaemia, and suppression of the renin-angiotensin-aldosterone axis [Stewart et al., 1988]. The diagnosis of AME was made, based on metabolic balance studies and the urinary steroid metabolite profile along with a prolonged half-life of 11α^3H-cortisol confirming the impaired inactivation of cortisol. These studies showed that the hypertension and biochemical abnormalities responded to pituitary-adrenal suppression with dexamethasone and on oral hydrocortisone supplementation (or dexamethasone withdrawal) the syndrome could be reproduced with kaliuresis,

sodium retention with corresponding changes in the plasma electrolytes, and suppression of the renin-angiotensin-aldosterone axis. Concomitantly the patient's weight rose and there was an increase in his blood pressure.

The above description relates to what is now known as Type-1 Apparent Mineralocorticoid Excess, being a defect in the 11ß-dehydrogenase, but not 11-oxidase. This is supported by finding a normal increase in plasma cortisol following oral administration of cortisone in addition to the characteristic urinary steroid metabolite profile and a prolonged half life of 11α^3H-cortisol [Stewart et al., 1988].

Recently Ulick and colleagues described 4 cases of what has become known as Type-2 Apparent Mineralocorticoid Excess [Ulick et al., 1989; Ulick et al., 1990] (Recently summarised by Mantero [Mantero et al., 1994]). These patients have an identical picture of cortisol dependent low renin hypertension with hypokalaemic alkalosis and suppressed aldosterone and DOC. They however have a normal ratio of cortisol to cortisone metabolites in urine. Proof of abnormal 11ß-dehydrogenation was obtained by finding a prolonged half life of 11α^3H-cortisol [Mantero et al., 1994], thus cortisol will occupy the renal mineralocorticoid receptor. In addition these patients have an impaired ability to convert orally administered cortisone to cortisol [Mantero et al., 1994], explaining the unchanged ratios of cortisol to cortisone in plasma and urine and their metabolites in urine.

1.4.2. Liquorice and the Kidney

Key to understanding the pathogenesis of AME, was recognition of the similarities between the type of hypertension induced by liquorice and the syndrome. It had been known for many years that abuse of liquorice caused classical mineralocorticoid hypertension [Salassa et al., 1962]. Suggested explanations included binding of glycyrrhetinic acid (the active component of liquorice see

Figure 1.7) to the kidney mineralocorticoid receptor [Armanini et al., 1983] but two facts contradicted this.

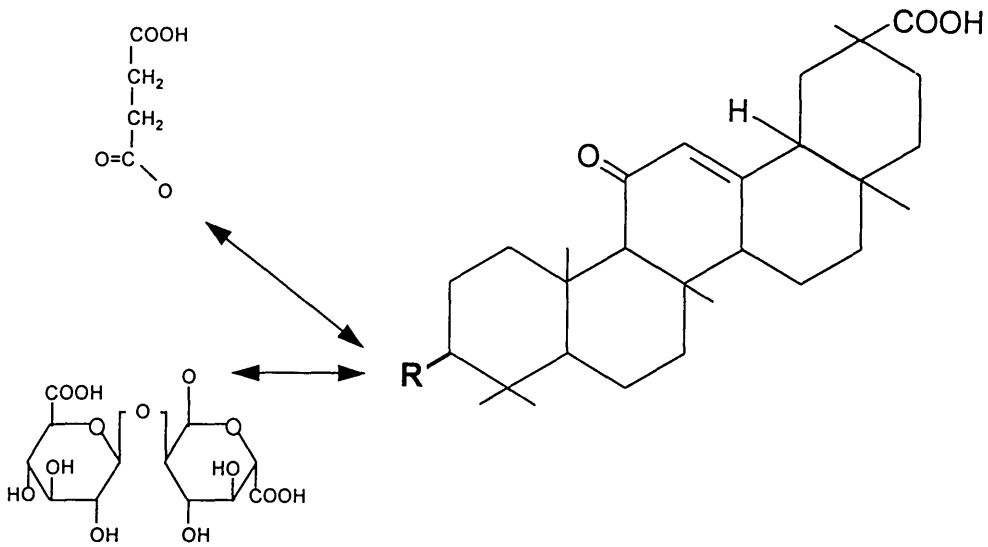


Figure 1.7 Inhibitors of 11ß-HSD

Note similarities in structure of 11ß-HSD inhibitors and steroids (Figure 1.2). To the right is the common triterpene structure. R is replaced by an OH group in glycyrrhetinic acid (GE - the principal active constituent of liquorice). GE is the hydrolytic product of the water soluble glycoside glycyrrhizic acid which also is present in liquorice (in GI, bottom left replaces R). The ulcer healing drug carbenoxolone (CBX) is a water soluble hemisuccinate derivative of GE (top left replaces R).

Firstly the affinity of the mineralocorticoid receptor for glycyrrhetinic acid was far lower than that for aldosterone, and secondly some elegant studies in both humans [Borst et al., 1953] and animals [Card et al., 1953] had shown that an intact adrenal gland was necessary to allow liquorice to exert its action. In fact oral cortisone was able to restore the effect of glycyrrhetinic acid in patients with Addison's disease [Borst et al., 1953] and dexamethasone produced natriuresis in subjects taking glycyrrhetinic acid [Hoefnagels & Kloppenborg 1983].

Further studies reproduced an AME like syndrome in subjects taking liquorice, confirmed by raised urinary excretion of 11ß-hydroxy metabolites of cortisol in comparison to 11-oxo metabolites (cortisone metabolites), sodium retention, kaliuresis, and a prolonged half life of 11α^{3}H-cortisol with suppression of the

renin-angiotensin-aldosterone axis [Stewart et al., 1987]. It therefore seemed that liquorice abuse resulted in an acquired form of AME [Edwards et al., 1993b]. Using the active ingredients of liquorice, glycyrrhetinic acid [MacKenzie et al., 1990] and glycyrrhizic acid [Kageyama et al., 1992], it was shown that the urinary steroid profile was identical to Apparent Mineralocorticoid Excess type 1 (as in liquorice abuse) but carbenoxolone did not result in a change of this ratio although total urinary cortisol metabolite excretion was reduced [Stewart et al., 1990b]. Carbenoxolone treatment thus was more like Apparent Mineralocorticoid Excess type 2.

Clinching the site of the defect, Edwards and Stewart [Stewart et al., 1987] looked at the effect of glycyrrhetinic acid on binding of glucocorticoids to rat renal receptors [Edwards et al., 1988]. It was shown by autoradiography that inhibition of 11ß-HSD in rat kidney with glycyrrhizic acid allowed ^{3}H-corticosterone to bind to aldosterone binding sites, whereas none bound in the absence of the 11ß-HSD inhibitor.

In-vitro work with purified Type-1 mineralocorticoid receptor [Krozowski & Funder 1983] as well as cloning and expression of the human Type-1 receptor [Arriza et al., 1987] has since shown it to have similar affinities for cortisol, corticosterone and aldosterone lending plausibility to the hypothesis that *in-vivo* an additional factor was necessary to ensure tissue mineralocorticoid specificity, namely 11ß-HSD. This hypothesis is obviously of paramount importance in a biological sense, challenging the simplistic view that receptor activation depended solely on ligand-receptor interaction.

Emphasising the importance of 11ß-HSD in the kidney, the bulk of plasma cortisone derives from the kidney [Whitworth et al., 1989b; Walker et al., 1992a]. Also, 11ß-HSD may play a role in the ectopic ACTH syndrome, where ACTH is thought to directly inhibit the enzyme in the kidney - causing the characteristic hypokalaemia. This was based on exceptionally high plasma cortisol/cortisone

ratios individuals with Cushing's syndrome, and a high cortisol/cortisone ratio in healthy volunteers infused with cortisol or ACTH [Walker et al., 1992a].

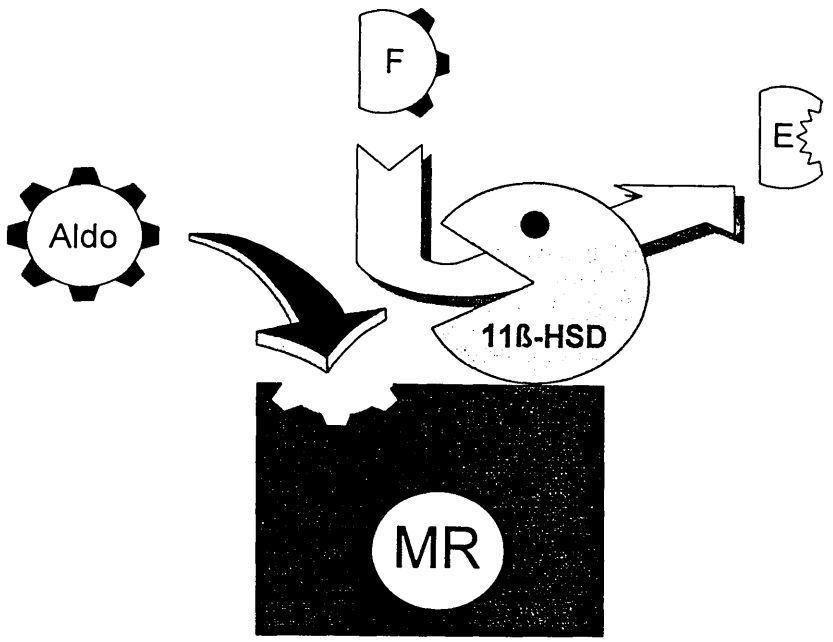


Figure 1.8 Enzyme Mediated Receptor Protection

The figure illustrates the concept of enzyme mediated receptor protection. The mineralocorticoid receptor (MR) is inherently non-specific with respect to cortisol (F) and aldosterone (Aldosterone) (and corticosterone). F is converted to E by 11ß-HSD allowing Aldo preferentially to bind to the renal mineralocorticoid receptor in spite of 100-1000 fold higher circulating levels of F.

Recently, several reports have confirmed the relevance of 11ß-HSD to mineralocorticoid receptor activation in a variety of other mineralocorticoid targets (including toad bladder mucosa [Brem et al., 1993], colonic mucosa [Whorwood et al., 1993a], and cultured collecting duct cells [NarayFejesToth & Fejes-Toth 1994]).

1.4.3. Isoforms of 11ß-HSD

The possibility of isoforms of 11ß-HSD was first noted by López Bernal in 1980 [López Bernal et al., 1980b], when he found that the *in-vitro* activity of the enzymes present in human trophoblast and decidua responded differently to

inhibitors and had different apparent K_m and specific activity. Later in 1985, the rat liver enzyme was found to have separable oxidase and reductase activities, achieved by means of phospholipases, detergents and alterations in pH [Lakshmi & Monder 1985], which was felt to be an indication of more than one type of 11ß-HSD. When considering the wide tissue distribution of this enzyme, its phylogenetic age (its presence in the toad bladder indicates evolutionary conservation for over 300 x 10^6 years [Gaeggeler et al., 1989]), the multiple effects of glucocorticoids, and the lack of substrate specificity, one has to say that it would be surprising if there was only one form of 11ß-HSD.

Could the current data fit with one isoform, the local environment determining tissue specific characteristics? In the liver, the enzyme's prevailing direction *in-vivo* appears to be opposite to that in the kidney [Bush et al., 1968; Stewart et al., 1988]. That conceivably could be brought about by differences in the redox state of the tissue involved and/or tissue specific differences in availability of NAD vs. NADP. Changes in pH is another possibility. The fact that *in-vitro* expressed 11ß-HSD [Agarwal et al., 1990] contained both activities actually supports this idea. I do however subscribe to the view, recently expressed by Monder and White [Monder & White 1993], that this is not likely to be the main explanation. The pH preferences of the liver and placental enzymes [Brown et al., 1993] for example are too discrepant and removed from physiological pH to be plausible regulators of enzyme direction. The same applies to the redox state.

Steroid or other hormones might regulate the activity as well as the direction of reaction in tissue and/or species specific manner. Thus the presence or absence (or α vs. ß position) of several functional groups (at positions different from 11) can determine whether in a particular tissue preparation, the direction of reaction is oxidative or reductive with regard to that particular steroid [Monder & White 1993]. Another example is the induction of placental 11ß-dehydrogenation by oestrogens in the baboon which appears to be species specific (not observed in humans, see later); 11ß-HSD appears to convert cortisone to cortisol at mid-

gestation, but at term the reverse [Pepe & Albrecht 1984b; Pepe et al., 1988]. Non-steroid hormones like thyroxine have also been shown to affect direction and activity of 11ß-HSD in tissue and species specific manner (for review see Monder & White 1993). But do we then have data supporting the view that intra- and inter-tissue differences in 11ß-HSD may not only be the result of variations in the intracellular environment but possibly due to post-translational modifications and/or represent products of separate genes? The answer to this question is yes.

The first clue came from the kidney. The initial theory was that the enzyme protected the renal mineralocorticoid receptor in a paracrine fashion as there was positive immunostaining for the enzyme in proximal tubules only, upstream from the classical mineralocorticoid receptor sites [Edwards et al., 1988]. As inhibition of 11ß-HSD had allowed glucocorticoids to bind to mineralocorticoid binding sites (distal tubule) [Edwards et al., 1988; Funder et al., 1988], this was somewhat peculiar, and later studies [NarayFejesToth et al., 1991; Bonvalet et al., 1990] confirmed bioactivity in the distal tubules, supporting the more plausible autocrine hypothesis. There were also several other characteristics (apart from not binding anti-sera raised against rat liver 11ß-HSD), of the kidney enzyme distinguishing it from the enzyme in other sites. Thus the affinity for its substrate was higher [NarayFejesToth et al., 1993; NarayFejesToth et al., 1994b; Stewart et al., 1994b], it utilises NAD rather than NADP as cofactor [Walker et al., 1992b; NarayFejesToth et al., 1994a], and is differentially regulated during ontogeny [Brem et al., 1994; Hundertmark et al., 1994; Maser et al., 1994] and by thyroid hormones [Whorwood et al., 1993b], and sex steroids [Low et al., 1993]. Recently, multiple mRNA species [Krozowski et al., 1990] and truncated 11ß-HSD forms [Krozowski et al., 1992] have been identified in the kidney although they seem to be functionally inactive when expressed *in-vitro* [NarayFejesToth et al., 1994a; Obeid et al., 1993].

The predominant placental isoform has very different K_m, pH optima, detergent solubility, lability, subcellular localisation, immunoreactivity and cofactor affinities

than the liver isoform [Lakshmi et al., 1993; Brown et al., 1993]. The amino acid sequence of the two is also very different [Brown et al., 1994]. Increasingly, it therefore seemed that there must be at least two genes encoding tissue specific isoforms. The liver predominant isoform has now been cloned in the rat [Krozowski et al., 1992; Moisan et al., 1992a], squirrel monkey [Moore et al., 1993], and man [Tannin et al., 1991]. The liver type cDNA encodes a reductase in intact mammalian cells [Low et al., 1994]. Several groups have been close to cloning a second gene in human placenta [Brown et al., 1993; Brown et al., 1994], human adult and foetal kidney [Stewart et al., 1994b; Stewart et al., 1994a], and cultured rabbit cortical collecting duct cells [NarayFejesToth et al., 1993; NarayFejesToth et al., 1994b]. However, Krozowski's group in Melbourne has recently announced the successful cloning of a cDNA for a 11ß-HSD-2 protein using expression cloning from human kidney [Krozowski 1994; Albiston et al., 1994]. Sequence alignment shows only 14% identity of 11ß-HSD-1 and 11ß-HSD-2 [Albiston et al., 1994].

The classical syndrome of AME (type 1) is almost certainly attributable to a defect in the 11ß-HSD-2 gene product, affecting only classical mineralocorticoid target sites. Recent studies reinforce this view, since no patients have displayed features of abnormal glucocorticoid receptor exposure and glucocorticoid receptor antagonists have no therapeutic effect, indicating that only mineralocorticoid receptors are involved in its pathogenesis [Speiser et al., 1993]. Thus, it is no longer surprising that no mutations in 11ß-HSD-1 were found in 4 patients with AME [Nikkila et al., 1993], although it should be remembered that the whole gene was not sequenced in these patients.

1.4.4. 11ß-HSD Beyond the Kidney

Outside the kidney, one of the earlier observations from our laboratory was the potentiation of action of topically applied glucocorticoids by co-application of glycyrrhetinic acid [Teelucksingh et al., 1990]; the skin vasoconstrictor assay, where skin pallor is observed following administration of glucocorticoids. Using mice, bioactivity was also shown in skin homogenates and immunoreactivity could be detected in the basal epidermal layers [Teelucksingh et al., 1990].

The above mentioned skin vasoconstrictor assay, is simply an estimate of dermal blood flow. This therefore, along with the observation in AME that although sodium retention occurs in the acute phase, blood pressure increase occurs only after a lag period [Stewart et al., 1988], implicated other sites (than the kidney) of 11ß-HSD expression which might play part in blood pressure regulation. Another stimulating observation was also at hand, namely that dexamethasone suppresses hypertension in a proportion of what otherwise would be considered patients with essential hypertension [Hamilton et al., 1979; Whitworth et al., 1989a].

In rat vasculature, positive immunostaining and mRNA expression for 11ß-HSD is found in the smooth muscle [Walker et al., 1991b; Takeda et al., 1994c; Kornel 1994]. The density of 11ß-HSD was also found to be higher in the appropriate locations, i.e. resistance vessels [Walker et al., 1991b]. It was of course well known from animal experiments (for review see Walker & Williams 1992) that glucocorticoids affect vascular responses to a variety of agents. When these observations were followed up in man *in-vivo*, healthy volunteers consuming liquorice had increased dermal vasoconstriction to cortisol, the dermal vasoconstriction being still more intense in a subject with AME type-1 [Walker et al., 1992c]. Individuals with essential hypertension similarly had more intense dermal vasoconstriction in response to topically applied glucocorticoid than controls and about 30% of the hypertensive individuals had a prolonged half-life of 11α-^{3}H-cortisol [Walker et al., 1991a]. Several reports on 11ß-HSD in animal

models of hypertension are now appearing. Thus 11ß-HSD activity and expression is normal in the kidney but reduced in the liver of the Bianchi-Milan hypertensive strain [Stewart et al., 1993] and in the blood vessels of Dahl salt sensitive rats [Takeda et al., 1994a; Takeda et al., 1994b] and the Spontaneously Hypertensive Rat (SHR) [Takeda et al., 1993].

Fitting nicely with the original suggestion that 11ß-HSD expression is only seen in tissues exhibiting aldosterone selectivity [Edwards et al., 1988], early studies on brain 11ß-HSD failed to show significant bioactivity in homogenates [Funder et al., 1988; Edwards et al., 1988]. This was however later refuted by studies where addition of the cofactor NADP to the homogenate resulted in detectable bioactivity in various regions of the brain, notably the cerebellum, hippocampus and cortex [Lakshmi et al., 1991; Moisan et al., 1990a; Moisan et al., 1990b]. This bioactivity was localised mostly to neurons by immunohistochemistry [Lakshmi et al., 1991], and *in-situ* hybridisation [Moisan et al., 1990a; Moisan et al., 1990b]. These observations along with the observations of Teelucksingh [Teelucksingh et al., 1990], indicated that not only did 11ß-HSD modulate access of glucocorticoids to the mineralocorticoid receptor (type I receptor), but also to the type II glucocorticoid receptor. Another support for this was the observation that expression of Na^+/K^+ATPase subunit mRNA in the colon can be regulated with glucocorticoids [Fuller & Verity 1990], and effect reproduced by carbenoxolone but only in the presence of glucocorticoids. Later, 11ß-HSD-1 bioactivity and mRNA expression was demonstrated in rat colon [Whorwood et al., 1992].

In addition to the established exogenous inhibitors of 11ß-HSD, glycyrrhetinic acid and carbenoxolone, several endogenous compounds are competitive inhibitors of 11ß-HSD in vitro, including progesterone derivatives and bile acids [Monder & White 1993; Latif et al., 1994]. It is an intriguing possibility that these act as endogenous regulators of enzyme activity and that they account for defective 11ß-HSD in some clinical syndromes, including the ectopic ACTH syndrome (see above). Inhibitory activity may also account for the steroid sparing effects of some

herbal remedies in disorders like asthma [Homma et al., 1994]. Enhancing activity has been claimed for angiotensin converting enzyme inhibitors, as part of their pro-natriuretic action [Riddle & McDaniel 1994]. Also, it has recently been suggested that 11ß-HSD may have a role in metabolising non-steroidal substances in the liver [Maser & Bannenberg 1994].

Morris and colleagues have found inhibitory activity for rat hepatic 11ß-HSD and 5ß-reductase in human urine extracted on silica columns (Sep-Paks) and eluted with methanol [Morris et al., 1992]. This combined action is especially interesting since an unexplained feature of AME is an associated impairment of 5ß-reductase activity. They attribute the inhibitory activity to "glycyrrhetinic acid-like factors" (GALFs), and report that their excretion is increased in normal pregnancy [Morris et al., 1992], heart failure and essential hypertension [Semafuko et al., 1993]. However, the control groups in their studies were poorly matched. Morris' method has been reproduced by our group, but we found that GALF excretion is not increased in ectopic ACTH syndrome, and is not regulated by ACTH in health [Walker et al., 1993]. Although GALF excretion is increased in pregnancy, no additional increase in hypertensive compared with normotensive pregnant women was found [Williamson et al., 1994]. Finally, no increase in GALF excretion in essential hypertension was found and no significant relationship between GALF excretion and indices of mineralocorticoid receptor activation (plasma potassium, renin activity, and aldosterone) or 11ß-HSD activity (half life of 11α^{3}H-cortisol, ratio of cortisol to cortisone in plasma and urine, and ratio of cortisol and cortisone metabolites in urine) [Walker et al., 1994].

Rare cases of hirsutism have been attributed to impaired 11ß-reductase activity, resulting in increased cortisol clearance with increased ACTH drive to adrenal androgen production [Phillipou & Higgins 1985]. These patients do not have a defect in renal sensitivity to cortisol and no mutation was demonstrated in the 11ß-HSD-1 gene in the one patient studied [Nikkila et al., 1993]. Given the above observation in hirsutism, it has been suggested that the hepatic conversion of

cortisone to cortisol could be defective in polycystic ovarian syndrome. In one study no abnormality of 11ß-HSD was demonstrated [Stewart et al., 1990a], but in a more recent study low ratios of cortisol to cortisone metabolites were documented in hirsute patients [Rodin et al., 1994].

There is now abundant evidence suggesting that glucocorticoids affect ovarian physiology [Inazu et al., 1990; Malbon & Hadcock 1988; Wang & Leung 1989; Adashi et al., 1981; Hsueh & Erickson 1978; Schreiber et al., 1982; Baldwin & Sawyer 1974; Jia & Hsueh 1990; Albiston et al., 1990]. Although Ghraf did not find 11ß-HSD bioactivity in the rat ovary [Ghraf et al., 1975], human ovaries have been shown to be able to convert cortisol to cortisone [Murphy 1981], and to contain mRNA encoding 11ß-HSD [Tannin et al., 1991]. More recently this has been confirmed by myself [Benediktsson et al., 1992] and Cooke and co-workers [Michael et al., 1993b; Michael et al., 1993a]. The latter showed glucocorticoids directly to inhibit LH-induced steroidogenesis in cultured granulosa lutein cells. This is modified by 11ß-HSD [Michael et al., 1993b].

Intriguingly the matter has been taken further by their subsequent finding [Michael et al., 1993a] that *in-vitro* fertilisation and embryo transfer patients that have detectable 11ß-HSD bioactivity in the cultured granulosa lutein cells are much less likely to become pregnant than those patients whose cells have no detectable bioactivity. The oocyte itself exhibits strong 11ß-HSD-1 immunoreactivity [Benediktsson et al., 1992]. This isoform is probably a reductase [Duperrex et al., 1993; Low et al., 1994; Moore et al., 1993], preferring NADP/H [Brown et al., 1993], in the oocyte promoting initial differentiation/maturation by increasing the availability of glucocorticoids and maybe suppressing local maternal immunological processes which at implantation would aim at destroying this partly foreign tissue. The granulosa lutein cells however prefer NAD, and thus possibly contain 11ß-HSD-2, which would be in keeping with the absence of

immunoreactivity (11ß-HSD-1) [Benediktsson et al., 1992]. For further discussion see Michael & Cooke 1994.

In the testis, glucocorticoids also inhibit LH-induced steroidogenesis, also modified by 11ß-HSD [Monder et al., 1994a]. In male rats, testicular 11ß-HSD activity increases at puberty [Phillips et al., 1989], spermatid 11ß-HSD increases during maturation [Neumann et al., 1993], and dominant masculine behaviour was associated with higher testosterone and higher testicular 11ß-HSD activity [Monder et al., 1994b].

1.4.5. 11ß-HSD in Pregnancy

The only study looking for 11ß-HSD in the umbilical cord failed to find any bioactivity [López Bernal & Craft 1981]. Murphy [Murphy 1977b], looked at enzyme bioactivity in freshly minced uterine myometrium, and found 11ß-HSD to be not only present, but the direction of activity to change as pregnancy advanced (oxidative in non-pregnant and early pregnancy, but reductive at term). Giannopoulos [Giannopoulos et al., 1982] was later unable to find any activity in human myometrium, although the latter study's methodology was comparable.

The same controversy has surrounded the question of the presence of 11ß-HSD in human foetal membranes [López Bernal et al., 1980b; López Bernal & Craft 1981; Giannopoulos et al., 1982; Murphy 1977a; Murphy 1981]. Some studies [Giannopoulos et al., 1982] found activity in chorion only, employing careful scraping to remove adherent decidua. Tanswell [Tanswell et al., 1977] found activity in both directions in freshly minced membranes from various stages of pregnancy, but the reductive part always dominating, with activity increasing towards term. Murphy had found membranous activity to be the same in both directions in early pregnancy but agreed with Tanswell on reduction being dominant at term [Murphy 1981]. This prompted Murphy to propose that membranes were an important source of cortisol for the foetus [Murphy 1977a].

López Bernal found 11ß-HSD activity in scraped or crude membrane preparations [López Bernal & Craft 1981], but demonstrated in studies on membranes from dichorionic twins [López Bernal et al., 1980a], that this activity could only be attributed to contamination of the membranes by decidua. Thus like amnion, the chorion itself is devoid of 11ß-HSD. This view is in good agreement with histochemical studies mentioned below.

Although cultured fibroblasts from human uterine tissue had been shown to be able to catalyse 11ß-ol oxidation in 1958 [Sweat et al., 1958], it was not until 1960 that bioactivity studies by Osinski [Osinski 1960] demonstrated the presence of 11ß-HSD in term placenta. Histochemical studies in the sixties, using the tetrazolium blue reaction [Hart 1966; Ferguson & Christie 1967] (pink or blue pigment deposit where oxidation of an hydroxy group occurs), examined 11ß-HSD type activity in the female genital tract. Two of these studies [Hart 1966; Ferguson & Christie 1967] failed to show 11ß-HSD in human term trophoblast or in foetal membranes, but found positive staining in the trophoblastic layer of the extraplacental chorion when using NAD as a cofactor. The third study found faint staining in the syncytium of term human trophoblast [Baillie et al., 1965] and no cofactor preference. The tetrazolium blue method's major drawback is that although it gives positive reaction where 11-dehydrogenation occurs, not all steroids are able to elicit this, even though they undergo 11ß-dehydrogenation. Thus it does not produce a positive reaction if the physiological glucocorticoid cortisol (or corticosterone) is used, and therefore investigators have resorted to using for example 11ß-hydroxyandrostenedione, as here above.

Osinki's findings [Osinski 1960] have since been confirmed by numerous others in a variety of animal species. The role of the placental enzyme has been thought to be protection of the foetus [Murphy et al., 1974; Dancis et al., 1978; Beitins et al., 1973; Dormer & France 1973] from the 2 - 10 times higher maternal glucocorticoid levels [Campbell & Murphy 1977], thus preventing glucocorticoid-mediated growth retardation [Reinisch et al., 1978; Katz et al., 1990; Benediktsson

et al., 1993] and possibly to allow the foetus to regulate its own hypothalamic-pituitary-adrenal axis [Pepe & Albrecht 1985].

The story regarding cofactor preference of placental 11ß-HSD has been rather confusing, some finding NADP preference [Blasco et al., 1986; Osinski 1960; López Bernal et al., 1980b] some NAD [Pepe & Albrecht 1984a] and some no preference [Meigs & Engel 1961]. It is of course plausible that there is more than one isoform of 11ß-HSD in the placenta, as described by López Bernal [López Bernal et al., 1980b] and Lakshmi [Lakshmi et al., 1993], although it now seems clear that the predominant isoform which is responsible for the barrier function is NAD-preferring [Brown et al., 1993; Benediktsson et al., 1993], and a product of a gene distinct from the liver type enzyme [Brown et al., 1994].

If the placental enzyme's function is to protect the foetus from maternal glucocorticoids, one would expect its direction to be oxidative, inactivating glucocorticoids. This seems to be the case at term in most species, being compatible with our knowledge that the foetus grows fastest during the last trimester [Langman 1981]. The various organ systems, however, grow and mature at different stages of pregnancy and there are well known interspecies differences in that respect. One can therefore expect variable effects of excess glucocorticoids, depending on the direction of enzyme activity during these "windows" of organ development. Therefore, one might expect important inter-species differences in the ontogeny and direction of placental 11ß-HSD. The data available so far seems to support the idea of inter-species differences. Pepe, Albrecht and co-workers [Pepe & Albrecht 1984a; Baggia et al., 1990a; Baggia et al., 1990b; Waddell et al., 1988a; Waddell et al., 1988b; Pepe et al., 1988; Pepe & Albrecht 1987; Pepe & Albrecht 1984b] have produced elegant studies in the baboon, where they injected radioactive glucocorticoids into pregnant animals, following the destiny of the steroids in both the mother and foetus. They subsequently manipulated their system with sex steroids and supplemented their *in-vivo* studies with *in-vitro* cell culture and bioactivity studies. In summary, they found that the prevailing

direction of enzyme activity changes during pregnancy, favouring reduction and production of active steroid at mid-gestation, but oxidation at term. This seems to be orchestrated by sex steroids, oestrogens inducing the conversion of cortisol to cortisone and repression being achieved by anti-oestrogens or foetectomy. The normally reductive enzyme at mid gestation could thus be made to become oxidative by treatment with oestrogens. At present the human story is similar with respect to term activity, placental 11ß-HSD being strongly oxidative [Blasco et al., 1986; Brown et al., 1993] and in the short term during labour, there is no change in *in-vitro* human placental 11ß-HSD activity [López Bernal et al., 1982b], thus always being strongly oxidative. In agreement with this was the *in-vivo* data of Beitins [Beitins et al., 1973], who injected cortisol and cortisone into the maternal circulation at term. They concluded that at term 25% of the foetal F and 90% of the foetal E is of maternal origin.

Contrasting with the baboon data, the human placenta seems to predominantly oxidise cortisol to cortisone at mid-gestation. This has been shown *in-vivo* using comparable methodology to that used in the baboon. Thus Pasqualini [Pasqualini et al., 1970] injected a mixture of cortisol and cortisone into the foetal circulation. Only 3 - 6% of the total radioactivity reached the maternal circulation and although interconversion of cortisol and cortisone took place in the placenta, little if any of the cortisol formed from cortisone in the placenta reached the foetal circulation again. Murphy also carried out an *in-vivo* study in humans at mid-gestation (13-18 weeks) [Murphy et al., 1974] and came to the same conclusion. Studies looking at *in-vitro* 11ß-HSD activity, in relation to stage of gestation, span week 7 to term [Blanford & Murphy 1977; López Bernal & Craft 1981; Giannopoulos et al., 1982] and find oxidation always prevailing. Thus the conclusion is that most of the cortisol crossing the human placenta is converted to cortisone and doesn't reach the foetus in a biologically active form, the placenta behaving as a barrier throughout gestation [Blasco et al., 1986].

It is important to keep in mind the fact that *in-vitro* studies may not be able to tell us about what happens *in-vivo*. For example the oxidative or the reductive part of 11ß-HSD may be more robust during extraction/homogenisation, giving a false results [Lakshmi et al., 1993]. Thus baboon 11ß-HSD *in-vitro* does always have predominant cortisol to cortisone conversion. This becomes more pronounced as pregnancy advances [Baggia et al., 1990a], contradicting the *in-vivo* perfusion data described above. Researchers have therefore turned to *ex-vivo* human placental perfusion, perfusing term isolated cotyledons. All the studies agree that at term oxidation is predominant [Levitz et al., 1978; Dancis et al., 1978; Addison et al., 1993; Addison et al., 1991]. The studies looking at the reductive component find little or no reduction [Dancis et al., 1978; Addison et al., 1993]. Problems associated with these studies include i) the use of concentrations of steroids that are high and clearly not physiological (μM) [Dodds et al., 1993; Levitz et al., 1978; Dancis et al., 1978] and ii) the use of non-physiological steroids [Addison et al., 1991]. Some authors (using μM steroid concentrations in recirculating systems) have suggested that other enzymes than 11ß-HSD contribute to the placental metabolism of glucocorticoids [Addison et al., 1991; Dodds et al., 1993].

From first principles one can imagine that there could be mechanisms other than 11ß-HSD affecting the transfer of active glucocorticoids across the placenta. Prime candidates are the plasma proteins albumin (low specificity, high capacity) and CBG (high specificity, low capacity Corticosteroid Binding Globulin). The two studies that looked at this came to different conclusions. Murphy examined steroid uptake into placental villi [Murphy 1979] employing various preparations, and concluded that albumin had virtually no effect on the transfer of cortisol into human placental trophoblast, whereas CBG combined with placental cortisol metabolism to prevent excessive maternal cortisol from reaching the foetus. Using placental cotyledon perfusion, Dancis *et al.* [Dancis et al., 1978] concluded that while neither CBG nor albumin had any effect on the extent of conversion of cortisol to cortisone, binding to albumin (but not to CBG) reduced the clearance of cortisol across the placenta.

1.5. PLACENTAL ANATOMY

Placental anatomy differs between species and its structure is therefore an important consideration when choosing experimental animals models. A brief description of rat and human placental anatomy follows but for a more comprehensive account, see Davies & Glasser 1968; Page 1993; and Boyd & Hamilton 1970. Placentas are classified according to the shape of the mature placenta and allantoic and yolk sac development, but in addition, according to the amount of maternal tissue erosion by foetal tissue, and the number of layers interposed between the maternal and foetal blood.

In both rats and humans, foetal tissue has eroded maternal tissue which is a major factor when classifying placentas. The anatomical classification introduced by Grosser in 1908 and 1909 classified placentas as i) epitheliochorial (no maternal tissue erosion following implantation), ii) endotheliochorial (partial maternal tissue erosion - maternal endothelium intact) and iii) haemochorial with complete maternal tissue erosion; maternal blood coming into direct contact with foetal tissue (rat and human).

The haemochorial placentas can be further subdivided according to the number of foetal trophoblast layers separating maternal and foetal blood. In humans this is only the syncytiotrophoblast giving rise to the term haemo-monochorial, whereas the rat placenta is haemo-trichorial. The shape of both the rat and human placentas is discoid, and the rat yolk sac is a permanent structure, whereas it is transient humans. No allantoic development is observed in humans and is only transient in the rat.

The human placenta (Figure 1.9) consists of a basal (closest to the myometrium) layer of maternal origin termed decidua, but the bulk (where foeto-maternal exchange takes place) is composed of villous foetal tissue within which are the foetal vessels. This part of the human placenta is called trophoblast and can be

divided into functional units (cotyledons), each served by a branch of foetal chorionic vessels. The blood supply of the placenta is dual. The two umbilical arteries carrying blood from the foetus, branch on entering the chorionic plate and branches of them in turn dive deep into the placental substance branching further as they enter smaller and smaller villi, in the end becoming capillaries. The vessels then return, having become venules, enlarging until veins emerge on the chorionic plate's surface. Thus on inspecting the chorionic plate one will see pairs of arteries and veins traversing the surface, at intervals disappearing into the placental substance. Each of these pairs will supply a cotyledon although anastomoses between cotyledons are common, both on the chorionic plate's surface as well as more distally.

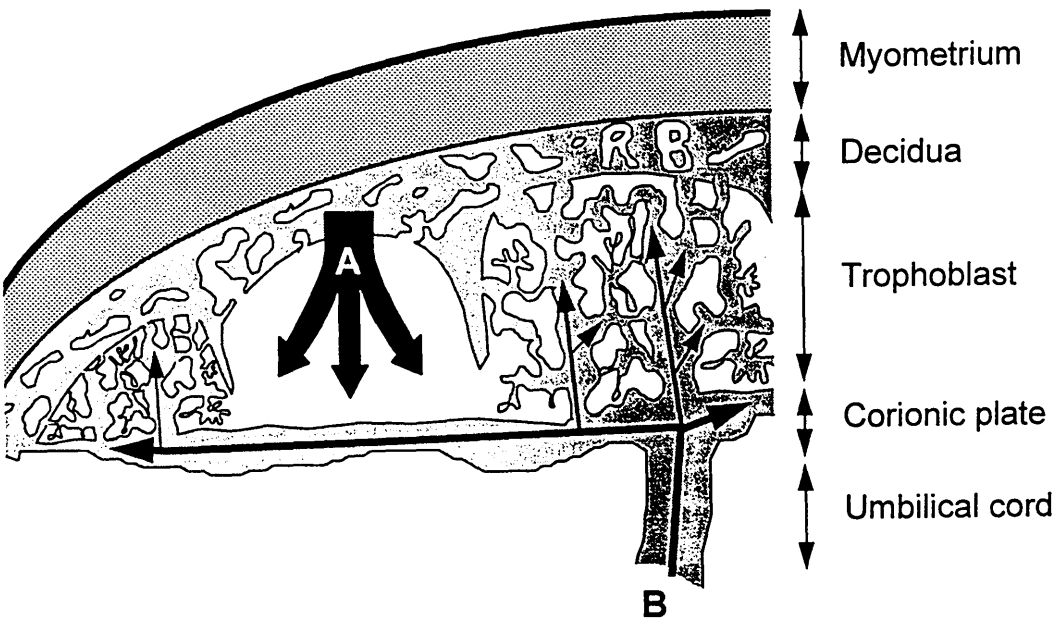


Figure 1.9 The Human Placenta

The figure shows a cross section of a part of a human placenta. Three idealised cotyledons are visible. The arrow A in the middle cotyledon shows the path of maternal blood spurting out of a spiral artery. The path of the foetal blood vessels is indicated by the branching arrow B entering the chorionic villi (trophoblast).

The trophoblast villi (chorionic villi) float in maternal blood which fills the intervillous space. The maternal blood enters the intervillous space via the decidual spiral arteries, bathes the villi and then drains back into the maternal circulation via venous openings in the periphery of the cotyledon. This arrangement is however not as simple as I have described it as cotyledons defined from the foetal "point of view" may have more than one supplying spiral artery and vice versa, cotyledons defined macroscopically by inspecting the decidual surface post-partum may contain various numbers of villous trees.

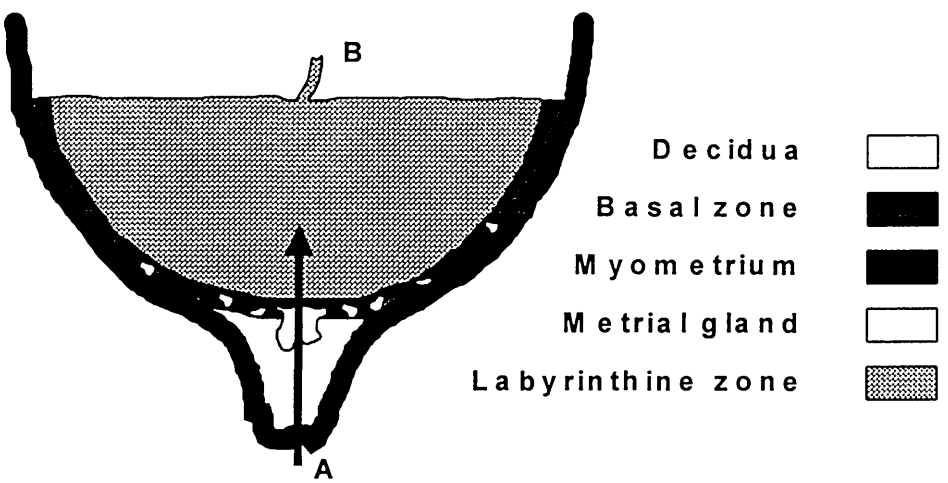


Figure 1.10 The Rat Placenta

A cross section through the a part of a rat uterus containing a placenta. The individual parts of the placenta as indicated. The maternal blood flow is via artery entering the mesometrial border of the uterus (A). Foetal blood enters the labyrinthine zone via the umbilical cord (B).

Although both rat and human placenta are discoid in shape, the rat placenta (Figure 1.10) is not divided into cotyledons. The part of the rat placenta corresponding to human trophoblast (i.e. where the foeto-maternal exchange takes place) is called the labyrinthine zone. The terminology is appropriate as the foetal vessels entering from the umbilical cord fuse and become a maze of interconnecting channels, thus

not forming villi as in the human placenta. The maternal blood arrives via a central artery through the basal zone and also travels along slits (channels) in the labyrinthine zone, separated by foetal tissue (having lost the maternal endothelium). In addition to the two zones of the rat placenta proper it also has third zone which may correspond to the human decidua but at term is reduced to a small lump in the centre of the basal zone, also extending into the fourth structure located in the mesometrial part of the uterus, called the metrial gland. The function of the metrial gland is unclear.

Thus, the rat placenta has to be considered a close match for the human placenta. Both are haemochorial, foetal tissue eroding maternal tissue completely. Only foetal tissue separates maternal and foetal blood although human placenta is haemomonochorial and the rat haemotrichorial (the 3rd rat layer is discontinuous [Davies & Glasser 1968]).

1.6. SUMMARY AND AIMS OF THE THESIS

Epidemiological data, from Professor Barker in Southampton and others, have shown that low birth weight is strongly predictive of deaths from ischaemic heart disease [Barker 1992b] as well as the development of several risk factors for ischaemic heart disease, including hypertension [Barker et al., 1993b]. The association between low birth weight and subsequent hypertension holds at all ages [Barker et al., 1989a; Law et al., 1991; Seidman et al., 1991; Barker et al., 1990; Law et al., 1993].

The strongest predictor for the development of hypertension is the unusual combination of low birth weight with high placental weight. The correlations with hypertension are continuous throughout the normal ranges of birth and placental weights, not merely apparent at the extremes of birth and placental weights [Barker et al., 1990]. The importance of these associations is emphasised by the fact that low birth weight is 3 times as powerful a predictor for the occurrence of ischaemic heart disease as smoking. The precise mechanism that might link prenatal events and later disease is not clear, although maternal malnutrition has been advocated [Barker et al., 1993a]. We have advanced the alternative hypothesis that glucocorticoid excess *in-utero* might be important [Edwards et al., 1993a].

The rationale for our hypothesis was existing data on glucocorticoid-induced foetal growth retardation both in humans and animals [Reinisch et al., 1978; Canavan & Goldspink 1988; Katz et al., 1990]. Furthermore, rats made diabetic with streptozotocin give birth to low weight offspring that also have high placental weights [Robinson et al., 1988; Canavan & Goldspink 1988]. These effects are only partially reversed by insulin treatment [Canavan & Goldspink 1988], and these animals have markedly raised maternal glucocorticoid levels [Heller et al., 1988]. By contrast, alloxan-induced diabetic rats have normal maternal glucocorticoid levels and do not exhibit this pattern of birth and placental weights

[Sybulski & Maughan 1971]. Exogenous glucocorticoids may increase or reduce placental weight, probably depending on dose [Gunberg 1957]. Of course glucocorticoids directly elevate blood pressure in adults and even *in-utero*, at least in foetal sheep as shown recently [Tangalakis et al., 1992]. We have demonstrated that treatment of pregnant rats with the synthetic glucocorticoid dexamethasone, in a modest dose which reduced average birth weight by 14% and did not alter litter size or gestation length, caused elevated blood pressures in the adult offspring, more than 5 months after exposure to exogenous glucocorticoid [Benediktsson et al., 1993]. The crucial question at this stage was twofold:

1. What controls foetal exposure to the much higher maternal glucocorticoid levels?

2. Does the glucocorticoid barrier vary under physiological conditions?

It has long been accepted that the foetus is protected from the 2 - 10 times higher [Campbell & Murphy 1977] maternal glucocorticoid levels by placental 11ß-HSD [Murphy et al., 1974; Dancis et al., 1978; Beitins et al., 1973; Dormer & France 1973]. This enzyme's presence in the placenta was first described in 1960 [Osinski 1960] and was then thought to be NADP dependent although subsequent researchers found either NADP [López Bernal et al., 1980b; Blasco et al., 1986; Osinski 1960], NAD [Bush et al., 1968] or no [Burton & Turnell 1968] cofactor preference. The significance of this was at first not realised but we now know that at least two 11ß-HSD isoforms exist [Seckl & Brown 1994] ; a low affinity, bi-directional liver-derived enzyme (11ß-HSD-1) and a less well-defined, high affinity, dehydrogenase (11ß-HSD-2) in the distal nephron [Mercer & Krozowski 1992; Stewart et al., 1994b; Krozowski 1994; Albiston et al., 1994] and also the placenta [Brown et al., 1993; Albiston et al., 1994].

The detailed distribution of 11ß-HSD in the placenta and its relationship to the foetal and maternal circulations was not known when I undertook this project, the only tools then available for looking at this (using *in-situ* hybridisation and

immunohistochemistry) were derived from the rat liver 11ß-HSD-1 [Agarwal et al., 1989; Monder & Lakshmi 1990] although we have in our laboratory very recently isolated an encoding cDNA for a placental 11ß-HSD-2 [Brown et al., 1994].

In summary, we postulated that a deficiency of placental 11ß-HSD could allow increased access of maternal glucocorticoids to the foetus, retarding growth and altering tissue development leading to subsequent adult disease. This has since been supported by studies showing that treatment of pregnant rats with inhibitors of 11ß-HSD results in low birth weight offspring that subsequently develop hypertension [Lindsay et al., 1994b; Lindsay & Seckl 1994]. In order to explore the above hypothesis it would be important to try and answer the following questions.

1. Is placental 11ß-HSD the glucocorticoid barrier, and if so, what is the relationship between placental 11ß-HSD activity and foetoplacental growth?

2. Are there species specific differences?

3. Is 11ß-HSD expressed pre-implantation?

4. What is the effect of toxins known to alter foetoplacental growth on the ability of placental 11ß-HSD to inactivate glucocorticoids?

5. Are any markers in cord blood able to tell us about foetal exposure to glucocorticoids - and, what is their relationship to placental 11ß-HSD efficiency?

2. MATERIALS AND METHODS

2.1. FLUID MEDIA

Krebs-Ringer Bicarbonate Buffer (KRB): This buffer was used for all *in-vitro* enzyme bioactivity studies on 11ß-hydroxysteroid dehydrogenase in both humans and animals (118 mM NaCl, 3.8 mM KCl, 1.19 mM KH_2PO_4, 2.54 mM $CaCl_2$, 1.19 mM $MgSO_4$, 25 mM $NaHCO_3$).

Human Placental Perfusate: The basis for the buffer used for perfusing the human placentas was also Krebs-Ringer bicarbonate buffer. It was modified for each separate circuit. For the "maternal" perfusion circuit, NaCl 119.9 mM, $NaHCO_3$ 21 mM, $CaCl_2$ 1.26 mM, KCl 12.6 mM, KH_2PO_4 1.18 mM, $MgSO_4$ 1.18 mM, Glucose 5.6 mM. The foetal circuit perfusate was the same but in addition contained Dextran 20 g/l (molecular weight 60000-90000, Sigma). The osmolality of this buffer was 294 mosmol/kg.

2.2. PREPARATION OF RADIOLABELLED MATERIALS

2.2.1. Tritiated Steroids

^{3}H-cortisone and ^{3}H-11-dehydrocorticosterone were prepared from [1,2,6,7-^{3}H]-cortisol (Amersham) and [1,2,6,7-^{3}H]-corticosterone (Amersham) respectively according to the following method.

Krebs-Ringer bicarbonate buffer was adjusted to pH 7.4 by bubbling with 5% CO_2 for 60 minutes. Using a Dounce tissue grinder, 1 g of fresh rat kidney cortex was homogenised in 20 ml Krebs-Ringer bicarbonate buffer containing 2 mM NAD.

The homogenate was then incubated for 2 hours at 37° C in a glass test tube with the desired amount of ^{3}H-cortisol or ^{3}H-corticosterone (typically 200 μl of stock dried down and resuspended in KRB). This was centrifuged for 15 min at approximately 1500 G. The supernatant and pellet separated were each extracted four times with 2 volumes of ethyl acetate by shaking vigorously for 10 minutes. The extracts were allowed to settle and the ethyl acetate evaporated to dryness under a gentle stream of nitrogen at 37° C. Dry residue were reconstituted in 2 ml ethanol and spotted onto TLC plates (250 μl per plate). The TLC plates were developed for 1 hour in 92% chloroform - 8% ethanol. Authentic cold standards (Sigma) for cortisone (or dehydrocorticosterone) were spotted on the plates as well. The area corresponding to cortisone (or dehydrocorticosterone) was then identified under UV-light (254 nm). The relevant areas on the TLC plates were scraped into glass vials and the steroids eluted overnight from the silica with ethanol. The ethanol eluates were combined for recovery and purity assessment.

The yield for ^{3}H-11-dehydrocorticosterone was 42% and purity checked with HPLC was > 93%. For ^{3}H-cortisone the yield was 39% and purity > 92%.

2.2.2. Iodinated Steroids

For cortisol and cortisone radioimmunoassays, iodinated labels were used. For the iodination of both, Cortisol-3-(O-carboxymethyl)oxime and Cortisone-3-(O-carboxymethyl)oxime had first to be synthesised. The cortisol-3CMO was made in-house by Dr. J. Corrie and cortisone-3CMO was made in-house by Mr. D. Burt. The method has been published [Al-Dujaili & Edwards 1987] but briefly, the relevant cold steroid was dissolved in methanol, carboxymethoxylaminehemihydrochloride and anhydrous sodium acetate added. After stirring for 2 hours at room temperature, the methanol was evaporated under nitrogen and spotted on TLC plates. The relevant mono-oxime was identified by the tetrazolium blue reaction [Neher 1964]. The steroid was purified by two further TLC separations.

The iodination itself was a modification of the histamine conjugation method [Chard 1987] and is routinely carried out in our department by Mr. D. Burt. The procedure was in four steps and identical for the synthesis of both ligands. The final product of the iodination is "steroid"-3CMO-^{125}I-histamine and will be abbreviated to ^{125}I-cortisol and ^{125}I-cortisone hereafter.

1. *Steroid activation:* Cortisol-3CMO (or cortisone-3CMO) was dissolved in dimethylformamide (DMF) to give 5.8 mg/ml. Fifty μl were cooled to 10° C, 10 μl each of tributylamine and isobutylchloroformate (diluted 1:60 and 1:110 with DMF respectively) added and kept at 10° C for 20 minutes.

2. *Histamine iodination:* Ten microlitres of histamine (0.22 mg/ml in phosphate buffer) were added to 1 mCi Na ^{125}I (Amersham). To this were added 10 μl each of chloramine-T and sodium metabisulphite (5 mg/ml and 12 mg/ml in phosphate buffer respectively). The mixture was vortexed and cooled on ice.

3. *Conjugation:* To the activated steroid mixture, 280 μl of cool (10° C) DMF was added and a 50 μl aliquot transferred to the ice cold ^{125}I-histamine preparation. Following addition of 10 μl 0.2 M NaOH the mixture was vortexed and incubated for 1 h on ice. The mixture was acidified by adding 1 ml of 0,1 M HCl and washed with 1 ml redistilled ethyl acetate. The ethyl acetate was discarded and the remaining mixture neutralised with 1 ml 0.1 M NaOH followed by addition of 1 ml KI (10 mg/ml in phosphate buffer).

4. *Extraction:* The iodinated steroid was then extracted by ethyl acetate (3 times) and the extract dried by adding a few crystals of Na_2SO_4. Purification was then by TLC and the relevant area identified by autoradiography. The band corresponding to iodinated steroid (of the usually 3 bands visible, one major band is used) is scraped off the TLC plate into glass vials and the steroid eluted with ethanol and stored at -20° C. The yield was typically 50 - 60%.

2.3. RADIOIMMUNOASSAYS

2.3.1. Cortisol

This direct second antibody radioimmunoassay, which was modified from McConway [McConway & Chapman 1986], used ^{125}I-cortisol (prepared in-house as detailed above) as ligand. Intra-assay CV was 5 - 11%. Inter-assay CV was 15.8%. Cross reactivity with cortisone of the second antibody used in this assay is < 0.1% according to the manufacturer (Scottish Antibody Production Unit - SAPU).

The assay diluent was 0.1 M citrate-phosphate buffer with 1% gelatine, adjusted to pH 4 with NaOH and/or phosphoric acid. Duplicate aliquots of plasma or serum (10 µl) were added to disposable LP4 (Luckhams) polystyrene tubes containing assay diluent (1:40 dilution of sample). Next the ligand was added (approximately 10000 cpm per tube) and then the first antibody (50 µl making 1:130000 final) which was "sheep anti-cortisol" (SAPU: Scottish Antibody Production Unit). The tubes were vortexed gently and incubated at room temperature for 4 h.

Then 50 µl of the second antibody (donkey anti-sheep/goat serum @ 1:15 initial; SAPU) and 50 µl non-immune sheep serum (@ 1:200 initial with 1 mM EDTA; SAPU) were added. The tubes were incubated at 4° C overnight and then spun at 4° C for 45 min at 1720 G. The supernatant was decanted to waste and the bound fraction counted in a γ-counter (LKB Wallac 1260 - counting efficiency of the 12 wells was 98.3% with SD of 1.5%). Cortisol (Sigma) standards were prepared in assay diluent and stored in aliquots at -20° C; duplicates were included in each run.

2.3.2. Cortisone

This radioimmunoassay was an in-house assay described by Whitworth [Whitworth et al., 1989b]. It was a second antibody radioimmunoassay using ^{125}I-cortisone (prepared in-house as detailed above) as ligand. As the antibody cross reactivity with cortisol was 3.5 - 4%, it was necessary to separate cortisol and cortisone before the radioimmunoassay with HPLC. Inter-assay CV was 4 - 6%. Intra assay CV was 11%.

The samples were pre-extracted with hexane (250 µl plasma diluted 1:2 with distilled water), centrifuged and then extracted with 5 volumes ethyl acetate. Following washing with 0.1 M NaOH the sample was evaporated to dryness under nitrogen. The sample was reconstituted in mobile phase (50% methanol:water - Rathburn Chemicals) and injected onto an HPLC system comprising an auto-injector (Waters 712 WISP), an HPLC pump (Waters 510), an absorbance detector (Waters 441) with 254 nm filter and a Waters reverse phase µ-Bondapak-C18 (3.9 x 300 mm) HPLC column. The flow rate was 1.3 ml/min. Retention time was checked at the beginning and end of each session with authentic cortisone standards (Sigma). The eluate corresponding to cortisone was collected. The eluate was reduced to less than half volume under nitrogen and extracted with 5 ml of ethyl acetate. Overall recovery was approximately 93%, while the HPLC recovery of cortisone checked with authentic tritiated cortisone (Amersham) was 97% - both were routinely checked for each batch of samples allowing accurate calculation of cortisone concentration in the original samples.

For the radioimmunoassay, duplicate 1 ml aliquots of the organic phase were pipetted into glass tubes (Corning 12 x 75 mm) and evaporated to dryness under nitrogen. The assay diluent was citrate/phosphate buffer with 0.1% BSA adjusted to pH 4 as for cortisol. Into each assay tube, 300 µl of assay diluent were added, whirlimixed and left for 15 min. Next the ligand (approximately 10000 cpm per tube) was added, followed by the first antibody (50 µl making 1:140000 final)

which was "rabbit anti-cortisol" [Whitworth et al., 1989b]. The tubes were vortexed gently and incubated at room temperature for 4 h. Then 50 μl of the second antibody (donkey anti-rabbit serum @ 1:10 initial; SAPU) and 50 μl normal sheep serum (@ 1:150 initial with 1 mM EDTA; SAPU) were added. The tubes were vortexed, incubated at 4° C overnight and then spun at 4° C for 45 min at 1720 G. The supernatant was decanted to waste and the bound fraction counted in a γ-counter (LKB Wallac 1260). Cortisone (Sigma) standards were prepared in assay diluent and stored in aliquots at -20° C; duplicates were included in each run.

2.3.3. Osteocalcin

This radioimmunoassay was performed by Dr. L. Tibi at the Department of Clinical Biochemistry, Royal Infirmary of Edinburgh. This was an in-house precipitated second antibody assay, based on the procedure described by Price [Price & Nishimoto 1980]. Purified bovine osteocalcin was used to raise the first antibody in rabbits and to prepare standards and iodinated ligand (chloramine-T method). The assay buffer composition was 20 mM Tris-HCl, 150 mM NaCl, 25 mM Na_4EDTA, 0.05% Tween-20, 0.02% sodium azide, at pH 7.2. Duplicate aliquots of plasma (50 μl) were incubated at 4° C for 24 h with ligand and first antibody. Following addition of the second antibody (normal rabbit serum/donkey anti-rabbit serum), the tubes were shaken, washed (Brij-35 in H_2O), centrifuged at 4° C for 30 min (3000 G) and the bound fraction counted. Intra-assay coefficient of variation (CV) was 5%, inter-assay CV was 12%.

2.4. In-Vitro Enzyme Assays

Tissues were obtained fresh and immediately placed on ice. All rats were Wistars. Ovaries were obtained from animals in proestrus. Animal tissues were homogenised manually with a Dounce tissue grinder. Human placenta was

homogenised using an Ystral mechanical homogeniser, employing three 10 second bursts of with 10 seconds of cooling on ice in between bursts. All tissues were homogenised in KRB, without BSA or glucose. For the enzyme assays, the protein content of the various tissue homogenates was determined using the method of Bradford, [Bradford 1976] using a kit purchased from Bio-Rad which had a detection limit of 20 μg protein per sample.

2.4.1. 11ß-Hydroxysteroid Dehydrogenase

The rat placental 11ß-HSD assay described below, was also used for human placentas and rat ovaries. It was a modification of the established "in-house" 11ß-HSD assay protocol. The description below relates to the assay of 11ß-dehydrogenase. For the reverse assay (11-reduction), NADH replaced NAD and ^{3}H-cortisone or ^{3}H-11-dehydrocorticosterone were substrates in place of ^{3}H-cortisol and ^{3}H-corticosterone respectively. Liver served as a positive control for the reductase assay, blanks were buffer only.

Incubations were carried out in duplicates or triplicates (12 x 75 mm glass test tubes - Corning Pyrex) with 0.5 mg/ml protein and a final concentration of 200 μM NAD and 12 nM ^{3}H-corticosterone (aliquot of stock dried down and reconstituted in KRB on day of assay) in KRB (0.2% BSA and glucose added) for 1 h at 37° C in a shaking water bath. The total incubation volume was 250 μl and incubation of buffer alone provided an assay blank. Blank rates with boiled homogenate ($>$ 60° C for $\geq$ 15 min) were no different for rat, human placenta or rat ovary. Rat kidney (known to have very high bioactivity) provided a positive control. The incubation was started by adding tritiated steroid and terminated by adding 2.5 ml of ethyl acetate.

The test tubes were whirlimixed thoroughly to extract the steroids, then allowed to settle and the organic layer removed and dried down. The residue was reconstituted in 100 µl ethanol, and along with cold standards (corticosterone and 11-dehydrocorticosterone) 40 µl spotted onto thin-layer chromatography plates (TLC plates: 20 x 20 cm Merck no. 5553) and developed for 1 h using a chloroform:ethanol solvent mixture (92:8 v/v respectively). The relevant regions were identified under UV-light (254 nm) and scraped into scintillation vials for counting (Packard ß-counter). The enzyme activity was expressed as the percentage conversion of ^{3}H-corticosterone to ^{3}H-11-dehydrocorticosterone, calculated from the radioactivity of each fraction: [($^{cpm\ product}/_{cpm\ product\ +\ cpm\ substrate}$) x 100]. Activity of blanks was always subtracted and net activity used for data analysis. Another way of expressing enzyme activity was to use moles of product formed during a fixed incubation period.

The above method using TLC plates and counting in a ß-counter was time consuming and also generated some quantity of airborne ß-emission due to scraping of the TLC plates. Therefore a new method was developed for extracting and separating the steroids. Slight variations in assay conditions are specified in the relevant Sections: variable NAD concentrations, time of incubation and protein content to ensure that the conditions used were on the linear part of the reaction progress curve with respect to the amount of enzyme added as well as incubation time to allow comparison between animals [Tipton 1992]. The substrate in all human experiments was the physiological human glucocorticoid cortisol (as opposed to corticosterone in the rat experiments). The separation and extraction of steroids was as detailed below.

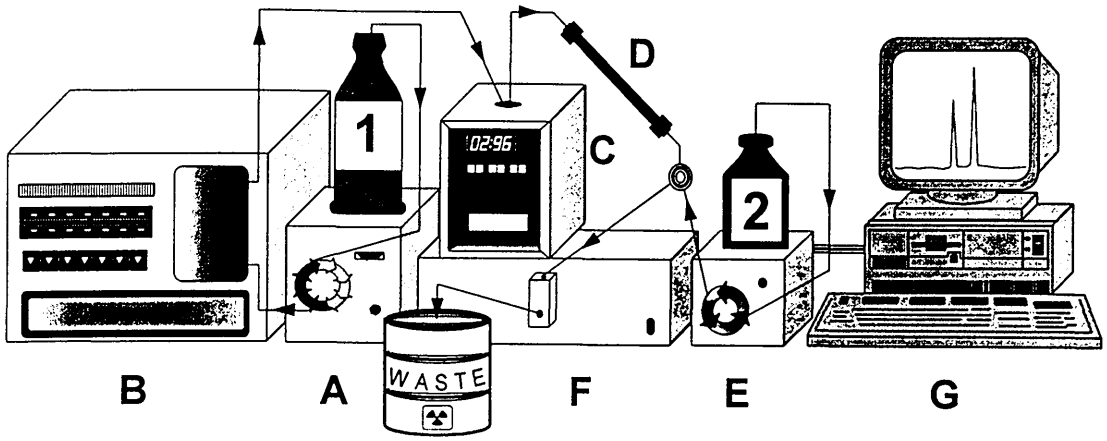


Figure 2.1 High Performance Liquid Chromatography Setup

The samples are placed in a carousel inside the autoinjector (B). The mobile phase is drawn from reservoir 1 by the HPLC pump (A), which drives the mobile phase through the autoinjector (B) where the sample enters the circuit. Having passed as the arrows indicate through the UV-monitor (C), the sample goes through the HPLC column itself (D) and is mixed with the scintillation fluid, which is drawn from reservoir (2) by a time controlled pump (E). The sample scintillates in the radioactivity monitor (F) before draining to waste as indicated. The setup is orchestrated by a computer (G) which records and stores the data from (C) and (F), allowing later analysis.

Following termination of the reaction by addition of ethyl acetate, the tubes were whirlimixed, allowed to settle and dried down as before, but now reconstituted in 600 μl HPLC grade methanol:water mixture (Rathburn Chemicals). An aliquot (200 μl) of the sample were then injected onto an HPLC column. The HPLC set-up (Figure 2.1), consisted of an auto-injector (Waters 712 WISP), an HPLC pump (Waters 510), an absorbance detector (Waters 441) set at 254 nm for detecting the elution profile of authentic cold steroid standards, a Waters reverse phase μ-Bondapak-C18 (3.9 x 300 mm) HPLC column linked to a radioactivity detector (Berthold LB506 C1), scintillation fluid pump (Berthold LB5035) and a computer for analysing and storing data (Berthold HPLC program v. 1.51). The flow rate for the mobile phase was 1.3 ml/min for separating cortisol and cortisone using 50% methanol:water, but 1.8 ml/min for separating corticosterone and 11-dehydrocorticosterone.

Details of intra- and inter-assay variation as well as recovery data are presented in the relevant results chapters under assay evaluation.

2.4.2. Alkaline Phosphatase and N-γ-L Glutamyl ß-Naphthylaminidase

Both assays were photometric assays, validated and carried out by Dr. T. Bramley (University Department of Obstetrics and Gynaecology, Centre for Reproductive Biology, Edinburgh). Both enzymes are stable on storage at -70° C. Briefly, the human trophoblast samples were homogenised as for 11ß-HSD with an Ystral mechanical homogeniser and an aliquot immediately frozen and stored at -70° C until assay. The protein content of the homogenates was determined as for 11ß-HSD.

The alkaline phosphatase assay was carried out in 13 x 100 mm polystyrene test tubes at 37° C for 35 minutes. Assay medium composition was 0.1 M piperazine-HCl, 5 mM magnesium acetate, 0.2% triton X-100, 0.01 M $ZnSO_4$. The substrate was p-nitrophenyl phosphate at 3 mM final concentration. Total assay volume was 1010 µl. The reaction was stopped by adding 3 ml of "stop solution" (20 g/l NaOH + 5 g/l EDTA). Optical density was read at 420 nm and results expressed as nmoles/min/mg protein.7777

The N-γ-L glutamyl ß-naphthylaminidase assay was carried out in 13 x 100 mm glass test tubes at 37° C for 3 h. Assay medium composition was 0.1 M tris-HAc, 5 mM EDTA, 100 mM glycylglycine. The substrate was N-γ-L glutamyl ß-naphthylaminidase at 0.4 mM final concentration. Total assay volume was 1025 µl. The reaction was stopped by adding 1 ml of 10% trichloracetic acid. Tubes were mixed gently and centrifuged at 3000 rpm for 10 min. 1 ml aliquot of the supernatant was incubated in 16 x 100 mm glass test tubes with 0.5 ml 0.2% sodium nitrite for 3 min. Then 1 ml of 0.5% ammonium sulphamate was added and incubated for further 3 min. Lastly, 2 ml of N-1-naphthyl ethylenediamine di

HCl (0.05%) were added and incubated for 45 min. Optical density was read at 580 nm and results expressed as nmoles/min/mg protein.

2.5. IMMUNOHISTOCHEMISTRY

This was done with technical assistance from L.P.Brett (Department of Pathology, Western General Hospital, Edinburgh). Adult Wistar rats (240 g) were deeply anaesthetised with pentobarbitone and perfused through the ascending aorta with 250 ml ice-cold saline followed by 500 ml ice-cold paraformaldehyde (4 M) in phosphate buffer (0.1 M, pH 7.4). Proestrus ovaries, term placenta and uterus were removed and post-fixed overnight. Sections (4 μm) were cut and immunostained as described by Moisan [Moisan et al., 1990a], using two separate polyclonal rabbit antisera (5-125 at 1:50 dilution and 5-126 at 1:100 dilution) raised against purified rat liver 11ß-HSD as described by Monder & Lakshmi [Monder & Lakshmi 1990] (the antibody was a gift from Dr. C. Monder of the Population Council, New York). Detection was by the peroxidase-antiperoxidase method [Sternberger et al., 1970], using reagents from DAKO. Control sections were immunostained using preimmune rabbit serum.

2.6. IN-SITU HYBRIDISATION

This was done with technical assistance from Dr. J.L.W. Yau (Department of Medicine, Western General Hospital, Edinburgh). Adult Wistar rats (240 g) were killed by cervical dislocation, the ovaries rapidly removed and immediately frozen on dry ice. 10 μm cryostat sections were mounted onto gelatine and poly-L-lysine-coated microscope slides and stored at -85° C. Tissue sections were post-fixed in 4% paraformaldehyde/0.1 M phosphate buffer and washed in 3 changes of 2 x SSC containing 0.02% diethylpyrocarbonate.

T3 RNA polymerase was used to transcribe a 598 bp ^{35}S-UTP-labelled antisense cRNA probe from Sty I-linearized pBluescript vector containing the 1,265 bp 11ß-HSD cDNA insert [Agarwal et al., 1989]. The probe was denatured and added at a final concentration of 10 x 106 cpm/ml to hybridisation buffer (50% formamide, 0.6 M NaCl, 10 mM Tris, pH 7.5, 0.02% ficoll, 0.02% polyvinylpyrrolidone, 0.1% BSA, 1 mM EDTA, 0.1 mg/ml salmon sperm DNA, 0.1 mg/ml yeast tRNA, 0.1g/ml dextran sulphate and 10 mM dithiothreitol) as described by Yau [Yau et al., 1991]. In brief, 65 μl aliquots of probe were pipetted onto sections and covered with a coverslip (22 x 50 mm) which was sealed with DPX. Slides were incubated overnight at 50° C.

Following hybridisation, coverslips were removed by rinsing 3 - 4 times in 2 x SSC at room temperature, sections treated with RNase A (30 μg/ml, 45 min at 37° C) and washed in reducing salt concentrations to a final stringency of 0.1 x SSC/14 mM 2-mercaptoethanol at 60° C. After dehydration in increasing concentrations of ethanol in 0.3 M sodium acetate, slides were dried in air, dipped in photographic emulsion (Ilford) and exposed in light-tight boxes at 4° C for 21 days before being developed (D19, Ilford) and counterstained with haematoxylin-eosin. Controls were hybridised with similarly labelled non-complementary "sense" RNA probes under identical conditions.

2.7. NORTHERN BLOTTING

This was done with technical assistance from Dr. S.C. Low (Department of Medicine, Western General Hospital, Edinburgh). Ovary, hippocampus, kidney and liver were rapidly removed from female Wistar rats (240 g) following cervical dislocation, snap frozen and stored at -85° C. Total RNA was extracted from each tissue by the acid guanidinium thiocyanate method, [Chomczynski & Sacchi 1987]. Approximately 20 μg of total RNA was fractionated on a 1% agarose-0.7 M formaldehyde gel and blotted onto nitro-cellulose (Hybond C extra, Amersham) by

capillary transfer overnight. Hybridisation was performed at 42° C overnight in 50% formamide with a random primed ^{32}P-labelled 11ß-HSD cDNA probe cloned from a rat liver cDNA library [Agarwal et al., 1989] consisting of the excised p11DH insert and the membrane washed to a final stringency of 0.2 x SSC (1 x SSC is 0.5 M NaCl and 0.015 M sodium citrate) 0.1% sodium dodecyl sulphate (SDS) at 60° C and exposed to Kodak XAR film for 2 days, as described by Moisan [Moisan et al., 1990b].

2.8. Ex-Vivo Placental Cotyledon Perfusion

2.8.1. General Methods and Equipment Setup

The method used and the construction of the perfusion chamber was based on the description by Schneider [Schneider & Huch 1985; Schneider et al., 1972]. Immediately following a normal delivery the placenta was put into a plastic bag, immersed in ice-water and carried to the laboratory which was within 2 minutes walking distance of the delivery room. Care was taken to avoid handling of the placenta at delivery and only gentle traction on the umbilical cord was used when aiding delivery. When in the laboratory, the placenta was floated in ice cold water (still protected from the water by a plastic bag), foetal surface facing upwards.

A suitable pair of chorionic vessels was identified, supplying a well defined and macroscopically intact cotyledon. The foetal vessels were cannulated, with custom made stainless steel cannulas (foetal input cannula internal diameter was 1 mm, outer diameter 2 mm and effluent or venous cannula internal diameter 1.6 mm while outer diameter was 3 mm), sutured in place and the foetal circulation started. Foetal flow rate was 6 ml/min, the perfusate being gassed with 95% N_2:5% CO_2 (BOC). This was accomplished within 21 min of delivery (mean 14.8 min, see Table 6.1, page 161). Occasionally when vessel anatomy required, collaterals to adjacent cotyledons were tied off.

The relevant cotyledon was isolated by cutting it from the rest of the placenta, allowing a generous margin to avoid fluid leaks. The cotyledon was turned around, the maternal surface now facing upwards. The cotyledon was then embedded between the two parts of a perfusion chamber which was custom made in the Medical Physics Workshop at the Western General Hospital, Edinburgh (Figure 2.2).

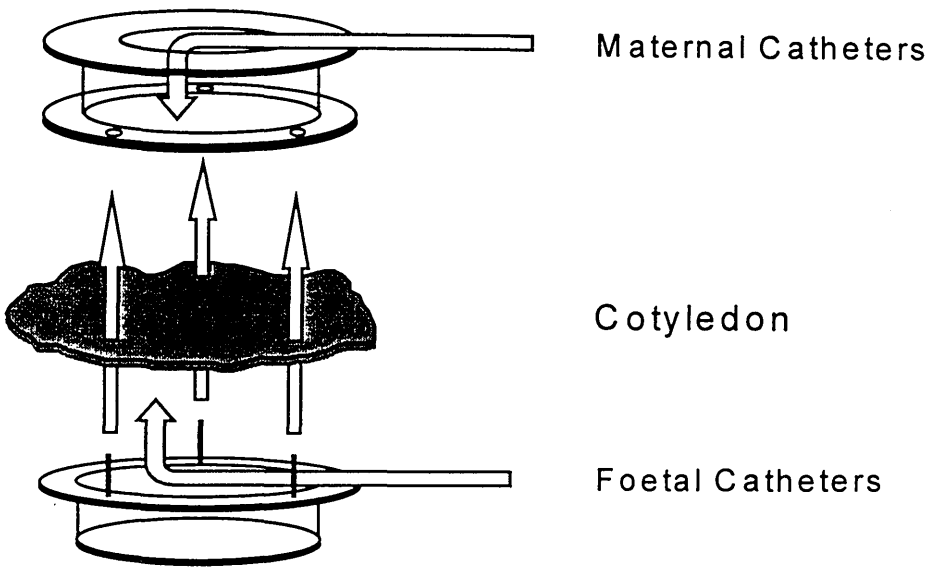


Figure 2.2 The Perfusion Chamber.

The chamber is made of transparent plexiglas (wall thickness 4 mm). The top and bottom parts are 8 cm wide and 5 cm high. The two parts are held together with pointed threaded pins (30 x 2 mm) securing the cotyledon between the two parts as indicated with the open arrows in the figure. The maternal and foetal catheters enter the chamber as indicated.

The top of the lower part was covered with "labfilm" to prevent sagging of the cotyledon, thereby preventing undue stretch on vessels and other tissues. The relevant area on the maternal side of the cotyledon was identified by virtue of slight blanching, and pierced to a depth of 1 - 2 cm (depending on the thickness of the selected cotyledon) using two 21G cannulas. The separate maternal circulation was started at 10 ml/min, the perfusate gassed with 95% O_2:5% CO_2 (BOC). The maternal circulation was established within 30 minutes of delivery (mean 23.3 min, see Table 6.1, page 161). Exit of the foetal perfusate was via the foetal circuit effluent catheter. Maternal outflow was via the venous openings in the periphery of

the cotyledon, collecting on top of the cotyledon (thus keeping it moist) inside the perfusion chamber, then siphoned to waste (Figure 2.3). The chamber itself was housed inside a temperature controlled incubator (Figure 2.3).

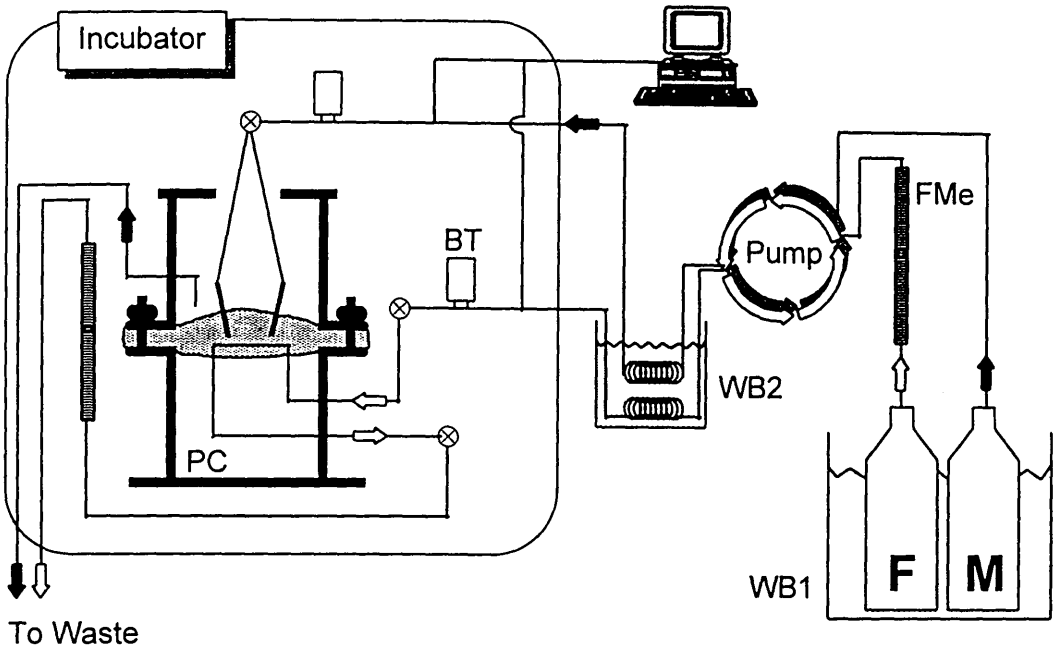


Figure 2.3 Setup Diagram

The diagram is not to scale. Arrows indicate direction of perfusate flow, driven by the multichannel peristaltic pump. PC: perfusion chamber, containing cotyledon, within temperature controlled incubator (Isolette®; Air Shields Europe). F: foetal reservoir, M: maternal reservoir. WB1 and 2: water baths. FMe: flow meter in foetal inflow. Another flow meter was located in foetal outflow allowing detection of fluid leaks. BT: bubble trap in foetal inflow. Another bubble trap was located in the maternal inflow. The computer continuously monitors temperature and pressure. Sampling ports (⊗) were located as close to the cotyledon as possible.

The two circulations were driven by a peristaltic pump (drive 503S with 8 roller 5 channel microcassette pumphead 308MC; Watson-Marlow). Different flow rates for the two circulations were achieved by using different bore tubing segments for the pump (maternal bore 2.54 mm, foetal bore 1.85 mm). Most types of tubing are very permeable to gases which necessitated special tubing materials for the two circuits. The tubing segment for the pump itself was Marprene II (Watson-Marlow) and the tubing for the rest of the circuits was Tygon (Watson-Marlow; bore 3.2 mm). The dead-space of the circuits was 28 ml and 31 ml maternal and

foetal respectively. To prevent bubble formation within the tubing, the perfusates were gassed after warming (WB1 in Figure 2.3). Bubble traps (custom made - plexiglas) were also employed. Due to a marked drop in temperature (4 - 5° C) on passing through the pump it proved necessary to include a second water bath before the tubing entered the incubator housing the perfusion chamber. Three way taps located as close to the cotyledon as possible allowed samples to be taken for analysis.

2.8.2. Monitoring of the Perfusion

Although human placental *ex-vivo* cotyledon perfusion has been in use for decades, different laboratories have used widely different methods to evaluate/validate their systems. Universally, authors claim suitability/success of their particular methodology. It appears that the placenta is a relatively sturdy organ in metabolic terms (although early hypoxic and/or perfusion related ultrastructural changes can be shown [Kaufmann 1985]), exhibiting remarkable tolerance to and ability to recover from the insult of delivery (evidence summarised by [Schneider 1991]). There is little agreement on which parameters should be used to monitor viability. Most researchers report lactate production, gas transfer (step-up across the foetal cotyledon), circuit pressure, and foetal circuit volume loss, but several other parameters like glucose consumption, lactate/pyruvate ratio, nucleotide concentration, morphology, transport of specific substances or production of placental proteins like ß-hCG are also used [Abramovich et al., 1987; Brandes et al., 1983; Challier 1985; Contractor & Stannard 1983; Hauguel et al., 1983; Illsley et al., 1984; Kuhn et al., 1988; Miller et al., 1985; Penfold et al., 1981; Wier & Miller 1985; Young & Schneider 1984]. It follows that it is difficult to compare results from different laboratories since methodologies differ in many respects: composition of the perfusion medium, flow rates and materno-foetal flow ratio, number of maternal cannulae, size of cotyledon perfused, etc.. The choice

of parameters to estimate viability in the series of perfusions presented in this thesis was as follows.

1. *Morphology:* Morphological changes are early and sensitive signs of inadequate perfusion due to either hypoxia or to high (> 60 mmHg) circuit pressure [Kaufmann 1985; Jauniaux et al., 1991]. Morphology is not compromised by using perfusate devoid of red blood cells for an extended period [Miller et al., 1985]. For the series of perfusions presented here, both light and electron microscopy were employed, trophoblast samples being taken before the start of perfusion, (i.e. within 21 min of delivery, see Table 6.1, page 161) and after the perfusion at 3 h from perfused as well as adjacent non-perfused tissue.

   For light microscopy, samples (approximately 0.5 cm in diameter) were fixed in formalin for a minimum of 24 h. The samples went through routine tissue processing at the Department of Pathology (Western General Hospital, Edinburgh) using Ames VIP automatic vacuum impregnation tissue processor. Briefly, the specimens went through various stages of impregnation with ethanol, xylene and paraffin before being embedded in Tissue Tek II wax. 3 - 4 μm sections were cut on a rotary microtome and picked up on a 50° C water bath onto poly-L-lysine coated slides. They were dried at 37° C for a minimum of 1 h and then stained with haematoxylin-eosin.

   For electron microscopy, specimens (approximately 1 - 2 mm in diameter) were immediately fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate/HCl buffer (pH 7.2 - 7.4) overnight at 4° C. The samples then went through routine processing at the Department of Pathology (Western General Hospital, Edinburgh) as follows. Specimens were washed in deionised water 3 times for 20 min. Secondary fixation was in 1% osmium tetroxide in deionised water for 45 min at room temperature. The specimens

were then dehydrated in 50%, 75%, 100% methylated spirits, each for 10 min and then in absolute ethanol for 3 x 10 min. Linking was with propylene oxide (10 min x 3) and impregnation in Emix resin (Fisons) overnight at room temperature. Polymerisation took place at 70° C for 18 - 24h.. 90 nm sections were cut, mounted on 300 mesh copper grid and stained with uranyl acetate/lead citrate. The microscope was Jeol 100CXII Transmission Electron Microscope operating at 60 kv. Photomicrographs were taken on Kodak 4489 film.

2. *Volume conservation (fluid leaks):* As crude indicators of fluid leakage, while the perfusion was running, two glass flow meters were used (Meterate L14X; Jencons), one in the foetal inflow and the other in the foetal outflow. If there was discrepancy between the two, the perfusion was abandoned. The average foetal output was also measured by collecting the foetal effluent over 5 min intervals, knowing that the input was 6 ml/min. Keeping foeto-maternal flow ratios between 0.3 - 3 seems important to minimise leakage [Schneider & Huch 1985]. *In-vivo* flow rates on both maternal and foetal sides have been estimated as being about 20 ml/min per 30 g lobule [Kirkinen et al., 1983]. This however will result in villous oedema in *ex-vivo* perfusion [Kaufmann 1985]. Foeto-maternal flow ratio here was 0.6.

3. *Metabolic activity:* As the primary indicator of constant metabolic activity throughout the perfusion, the activity of 11ß-HSD was followed for an extended period. Lactate generation was also looked at by sampling the foetal effluent, looking for a fall in the lactate output following establishment of the perfusion. Thus, perfusate samples (1.5 ml) were taken periodically from the foetal effluent, placed into pre-weighed glass tubes containing 5 ml of cold (4° C) 5% perchloric acid and immediately refrigerated. The analysis was carried out in the Department of Clinical Biochemistry, Royal Infirmary of Edinburgh by Dr. S. Walker. The lactate concentration was measured using a Cobas Bio (Roche) centrifugal analyser, monitoring the rate

of reduction of NAD to $NADH^+$ fluorimetrically at an excitation wavelength of 340 nm in 0.5 M glycine buffer (pH 9.6) in the presence of 0.2 M hydrazine hydrate and lactate dehydrogenase. Lactate standards were used. The perfusate concentration was calculated after correcting for dilution with the perchloric acid. The sensitivity was 0.2 mM lactate (translates into minimum production rate detectable of 32 μmol/min/kg), CV was 6 - 8%.

Other indicators of metabolic activity employed were CO_2 generation and pH, both in the foetal input and output. Both were analysed with an arterial blood gas analyser (pH Blood Gas Analyser 213 with Temperature Controller 227; Instrumentation Laboratory). The precision was $\pm$ 0.003 pH and $\pm$ 0.5 mmHg for pCO_2. Although oxygen consumption frequently is used as an estimate of metabolic function in a variety of experimental set-ups, it has been criticised for giving potentially misleading information in placental perfusion systems. Thus measured (apparent) O_2 consumption has been reported in the absence of tissue [Penfold et al., 1981], using conventional methodology, and Kuhn [Kuhn et al., 1988] reported very low O_2 consumption (compared to several other researchers) in a series of experiments where morphology was conserved as was transfer of antipyrine.

4. *Circulatory Overlap:* Following cannulation of the foetal circuit, slight blanching of the maternal surface could be observed which guided as to the area to insert the maternal cannulae. The insertion was not via the spiral arteries (which was abandoned as it proved to be very difficult technically and time consuming [Schneider et al., 1972]) and therefore slight inequalities in overlap of the maternal and foetal circulations are unavoidable. The extent of overlap was monitored by looking at O_2 transfer across the placenta, gassing the two circuits with different composition gasses (95% O_2 on the maternal side and no oxygen for the foetal perfusate), step-up of pO_2 from the foetal input to the foetal effluent indicating overlap of the circulations. The pO_2 was measured with an arterial blood gas monitor (pH Blood Gas

Analyser 213 with Temperature Controller 227; Instrumentation Laboratory, precision ± 1 mmHg). If no oxygen was delivered via the maternal perfusate or if there was inadequate overlap of the circulations, a step-down in pO_2 was observed.

5. *Pressure and Temperature:* Foetal perfusion pressure > 60 mmHg has been reported to cause diffuse damage to foetal villi during *ex-vivo* perfusion [Jauniaux et al., 1991]. Pressure transducers (Elcomatic EM 750A) inserted in the afferent section of each circuit continuously recorded pressure and were linked to a computer program written by the Department of Medical Physics (Western General Hospital, Edinburgh). Sampling rate was 1 Hz. Temperature was simultaneously recorded continuously in both circuits (afferent arm) by virtue of in-line K-type thermocouples connected to a digital thermometer (Fluke 52 K/J; resolution 0.1° C, accuracy 0.1% of reading + 0.7° C). The perfusion was considered unsuccessful if pressure in the foetal circuit exceeded 60 mmHg.

2.8.3. Comparison of Results and Sample Preparation

Comparison of results: A well recognised problem with placental cotyledon perfusions is comparison of results between laboratories and indeed also within laboratories. To enable that comparison, a reference molecule is usually included in the perfusion. The most commonly used reference substance is antipyrine, a small inert lipid soluble salt. Antipyrine is a model for fast and flow dependent transport (diffusion). In order to compare the results here with previous publications on steroid transport and metabolism using *ex-vivo* perfusion, ^{14}C-Antipyrine was included in the maternal perfusate.

Sample preparation: Perfusate samples for analysis of steroid and antipyrine concentrations were collected from the foetal effluent over either 3 or 5 minutes. Initial perfusions were performed with cold steroid only and the foetal effluent

samples extracted by mixing with two volumes of ethyl acetate, shaking vigorously for 10 min (an adaptation of the extraction following the *in-vitro* 11ß-HSD assay as detailed above). The samples were allowed to settle and the organic phase removed, evaporated to dryness before reconstituting in 600 µl of mobile phase (50% methanol:water; Rathburn) and analysed on the same HPLC system described in Section 2.4.1. 200 µl of each sample were injected, the elution time determined by comparison with authentic cold cortisone and cortisol standards (Sigma).

As for tritiated or ^{14}C-labelled substances, the amount contained in a peak detected with the UV-method (see above) could be deduced by calculating the area under the curve, using the computer program. To transfer the UV-units into meaningful values, a set of standards with known amount of the steroid of interest was included in each HPLC run. Each standard was injected in duplicate and a linear regression line fitted (least squares) to allow calculation of steroid concentration in the unknown samples. The detection limit of the system for cortisol was 2.5 x 10^{-12} moles injected. By collecting the foetal effluent over a minimum of 3 min, and concentrating to 600 µl (injecting 200 µl), peaks several orders taller than 2 times the variation in baseline were obtained.

Although some data could be obtained using the above method, there were several problems associated with it. Firstly, the typical volume of the perfusate samples was 30 ml which meant using 60 ml of ethyl acetate per sample, too large a volume to handle with ease. Secondly, there were numerous unidentified substances that travelled with the steroids in the lipid soluble phase and interfered with the UV absorption in such a way that the elution profiles from up to 50% of the samples were uninterpretable. Therefore, trace amounts of tritiated steroid (< 0.5% of the cold concentration) were used along with cold steroid and similarly ^{14}C-antipyrine (as opposed to cold) as a reference substance. Thus it was possible to measure the amount of steroid transferred as well as that of antipyrine in one and the same sample with the HPLC system set for dual-label estimation.

The amended method for preparing the samples (used for all but the very first perfusions), was modification of a method described for extracting steroids from plasma samples [Cannell et al., 1982], but using Sep-pak Plus® cartridges (Waters) and ethyl acetate for elution. Following collection, the perfusate samples were centrifuged for 5 min at 2500 - 3000 rpm and the supernatant decanted to fresh containers to get rid of debris and red blood cells. As the samples were stable on freezing they were either frozen at -20° C or the sample preparation completed as follows.

First the samples were acidified to pH 2 using HCl (checked with pH paper). The Sep-pak Plus® cartridges were mounted on a vacuum manifold (Super Separator-24; Amersham). The cartridges were preconditioned with 5 ml ethyl acetate followed by 5 ml H_2O (10 ml if using Sep-pak Plus® for the second time - no difference in extraction recovery as compared to first extraction - n = 12). Next the samples were loaded followed by a wash with 5 ml H_2O. Finally, elution was with 5 ml ethyl acetate. Care was taken not to let the vacuum exceed 15 inHg, and to keep the flow rate steady, not exceeding 30 ml/min. The eluate was evaporated to dryness using a sample concentrator, at less than 40° C under a gentle stream of air. The dry residue was reconstituted in 600 μl mobile phase (50% methanol in water) for 10 min in a water bath at 37° C. The tubes were carefully whirlimixed and centrifuged ($\approx$ 2500 rpm) and the samples transferred to small HPLC-vials for injection (200 μl) onto the HPLC column. The flow rate of the mobile phase was 1.3 ml/min and run time 15 min per sample. Retention (elution) time was checked with standards of cortisol and cortisone (Sigma) included in the beginning and at the end of each session.

3. RAT OVARIAN 11ß-HSD

3.1. INTRODUCTION

There is now abundant evidence suggesting that glucocorticoids affect ovarian physiology. Thus systemic administration of exogenous glucocorticoids has been shown to reduce ovarian weight [Inazu et al., 1990] and inhibit ovulation [Baldwin & Sawyer 1974]. Although the inhibition of ovulation has been thought to reflect glucocorticoid actions at the anterior pituitary, where they inhibit the preovulatory LH surge [Baldwin & Sawyer 1974], the presence of glucocorticoid receptors [Schreiber et al., 1982] and glucocorticoid-responsive gene products [Malbon & Hadcock 1988; Albiston et al., 1990] in the ovary, suggests that direct effects also occur.

In support of this, glucocorticoids have been shown to inhibit FSH-stimulated aromatase activity [Hsueh & Erickson 1978] and augment progesterone accumulation [Adashi et al., 1981] in granulosa cells *in-vitro*. Similarly, glucocorticoids stimulate the production of plasminogen activator by isolated granulosa cells [Wang & Leung 1989; Jia & Hsueh 1990], an action thought important in ovulation control [Reich et al., 1985].

In the male, systemic excess of cortisol like in Cushing's syndrome reduces sexual function and one of the responses to stress in the male is a reduction in sex drive. During stress, for example in subordinate primates, systemic glucocorticoid levels are raised in conjunction with reduced testosterone levels [Sapolsky 1985] (also observed in human stress [Kreuz et al., 1972]). These could possibly be central effects, but direct effects of glucocorticoids on the testis might also be responsible. Thus, just like the female gonads, male gonads contain glucocorticoid receptors [Evain et al., 1976], and cortisol has been shown to directly inhibit testicular

steroidogenesis [Bambino & Hsueh 1981]. It is therefore not surprising that 11ß-HSD has been found in this glucocorticoid target tissue. Already in 1965, the presence of 11ß-HSD was noted on immunohistochemistry (tetrazolium blue) [Baillie et al., 1965], and in 1989, Monder's group [Phillips et al., 1989] showed its presence in the rat testis interstitium using a specific antibody developed in rabbits against purified rat liver 11ß-dehydrogenase. In this study, testis 11ß-HSD appeared to be developmentally regulated, being absent in the foetus, and not appearing postnatally until at day 26, paralleling the postnatal increase in Leydig cell numbers and maturation [Haider et al., 1990]. In a later study [Monder 1991], bioactivity and immunoreactivity were found to co-exist in the rat testis, but bioactivity only and not immunoreactivity could be demonstrated in the epididymis, raising of course the possibility of 2 isoforms.

Thus by analogy with the testis, 11ß-HSD could be expected to be found in the ovary. Previous studies have not found 11ß-HSD bioactivity [Ghraf et al., 1975] or immunoreactivity [Phillips et al., 1989] in the rat ovary, but in contrast, human ovaries have been shown to be able to convert cortisol to cortisone [Murphy 1981], and to express mRNA encoding 11ß-HSD [Tannin et al., 1991]. Therefore, this study was undertaken, re-examining 11ß-HSD bioactivity and messenger RNA expression in the ovary of the rat.

3.2. METHODS

The methods employed have been detailed previously (Section 2), but will briefly be reiterated here. All tissues were obtained from animals in prooestrus. For 11ß-HSD activity, ovaries were homogenised in KRB, incubated with ^{3}H-corticosterone and NAD at 37° C. Steroids were extracted using ethyl acetate, followed by separation and quantification by HPLC. Animals were lethally perfused with paraformaldehyde and ovaries post fixed for immunohistochemistry but for *in-situ* hybridisation and northern blotting, fresh rat ovaries were immediately at surgery

frozen on dry ice. Cryostat sections were mounted onto slides and post fixed for *in-situ* hybridisation. An antisense cRNA probe was transcribed from a vector containing 11ß-HSD cDNA insert [Agarwal et al., 1989] and hybridised to the sections. Controls were hybridised with similarly labelled (^{35}S-UTP) non-complementary "sense" RNA probes under identical conditions.

For northern blotting, total RNA was extracted from each tissue by the acid guanidinium thiocyanate method, [Chomczynski & Sacchi 1987]. Following fractionation on agarose gel and blotting onto nitro-cellulose, hybridisation was with a cDNA probe derived from the same rat liver library [Agarwal et al., 1989]. For immunohistochemistry, detection was by the peroxidase-antiperoxidase method [Sternberger et al., 1970], using an antibody against rat liver 11ß-HSD raised in rabbits, kindly donated by Dr. C. Monder [Monder & Lakshmi 1990]. Preimmune rabbit serum served as control.

3.3. RESULTS

3.3.1. Immunohistochemistry

Consistently strong positive immunostaining was found in the oocyte (Figure 3.4). Additionally, positive immunostaining of moderate or low intensity was detected in cells comprising the luteal bodies (not shown). This varied between luteal masses, rather than within a given luteal body, presumably reflecting their varying stages of maturation. No staining was observed in the granulosa cells, theca cells, stroma or other ovarian components. No staining of any ovarian subregion was demonstrated with preimmune serum.

3.3.2. mRNA Expression

Northern blotting was used to detect mRNA expression and a single band hybridising to the 11ß-HSD probe (cDNA derived from rat liver 11ß-HSD) was detected in ovarian total RNA which corresponded to the major 11ß-HSD mRNA species demonstrated in rat liver and hippocampus. This was in contrast to the multiple bands found in kidney (Figure 3.1). *In-situ* hybridisation was used to localise 11ß-HSD mRNA expression to oocytes and also to a variable extent to luteal bodies, varying between luteal masses rather than within individual bodies and paralleling the immunohistochemical findings (Figure 3.5).

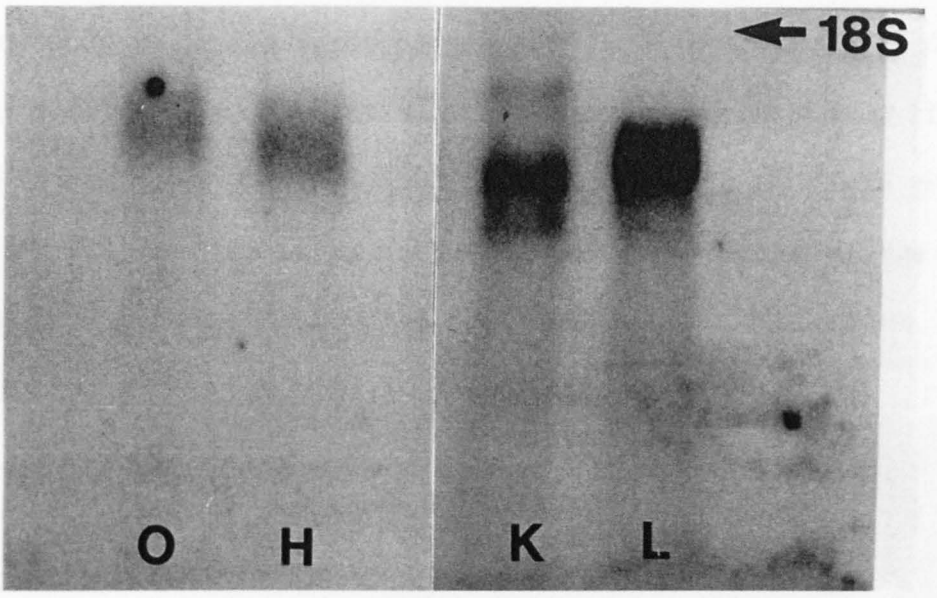


Figure 3.1 Rat Ovary: Northern Blotting.

This is an autoradiograph of total RNA hybridised with ^{32}P-labelled cDNA probes to 11ß-HSD mRNA. O = ovary, H = hippocampus, K = kidney, L = liver. Note multiple hybridising species in kidney but only one ovarian band corresponding to the one found in hippocampus and liver.

3.3.3. Bioactivity

11ß-HSD activity in the dehydrogenase direction was found in all ovarian homogenates using NAD. The time curves for the different protein concentrations (Figure 3.2) indicate linearity with respect to time for up to 10 minutes at both cofactor concentrations.

At protein concentration of 0.25 mg/ml incubated for 10 minutes, ovarian activity was 10% of kidney activity (ovary, n = 3: 179.6 ± 30.5 fmoles product formed, kidney, n = 2: 1742.4 ± 44.1 fmoles product formed). Increasing protein concentration produced a linear increase in fmoles 11-dehydrocorticosterone produced up to 0.5 mg/ml, beyond which a decrease in activity was observed (Figure 3.3).

No conversion of ^{3}H-11-dehydrocorticosterone to ^{3}H-corticosterone (reductase) was found using 200 μM NADH as cofactor. Multiple attempts to assay 11ß-HSD activity in freshly harvested rat oocytes from superovulated animals (a gift from Dr. J. Mullins, Centre for Genome Research, University of Edinburgh) as well as in 1 and 2 cell embryos (with both NAD and NADP) were without success.

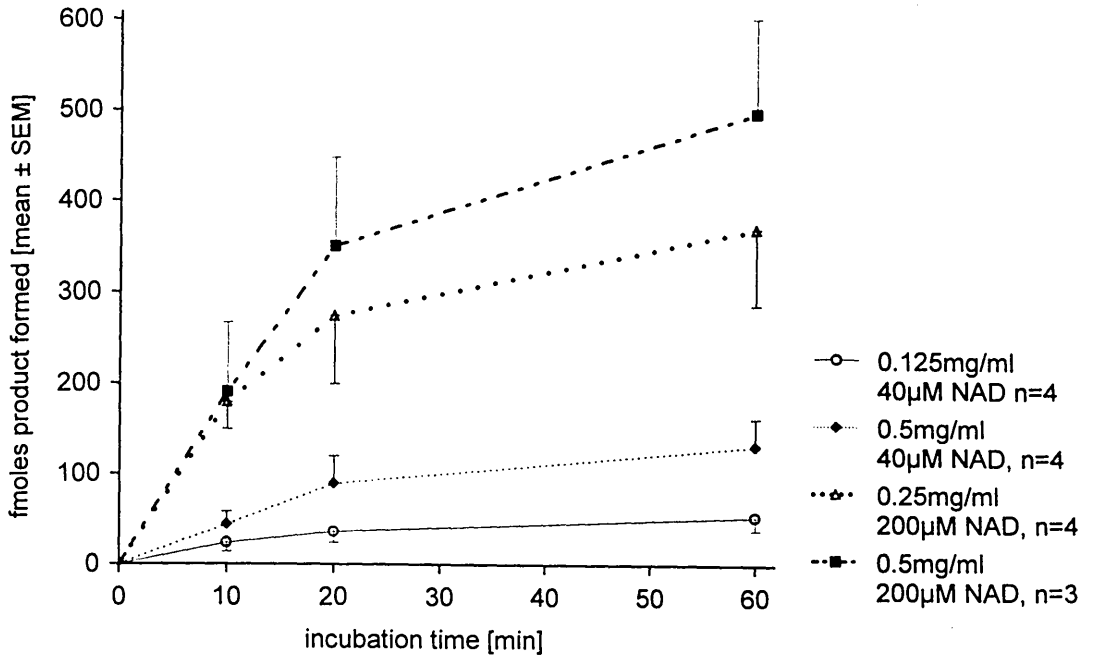


Figure 3.2 Rat Ovarian Bioactivity I: Time Course

11ß-HSD bioactivity (as fmol ^{3}H-dehydrocorticosterone formed from ^{3}H-corticosterone) in ovarian homogenates at three different protein concentrations and two different cofactor concentrations. Each point represents mean ± SEM from 3 - 4 animals (each incubated in duplicate).

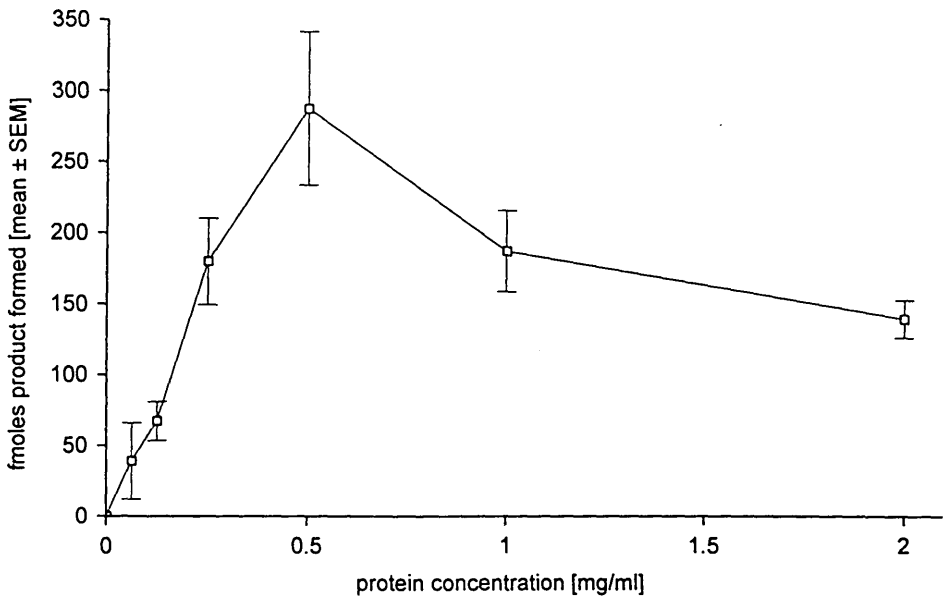


Figure 3.3 Rat Ovarian Bioactivity II: Protein Concentration.

11ß-HSD bioactivity (as fmol 3H-dehydrocorticosterone formed from 3H-corticosterone) in ovarian homogenates with increasing protein concentrations. Incubation time was 10 min, cofactor was NAD at 200 µM, substrate at 12 nM final. Each point represents mean ± SEM from 2 - 4 animals (each incubated in duplicate).

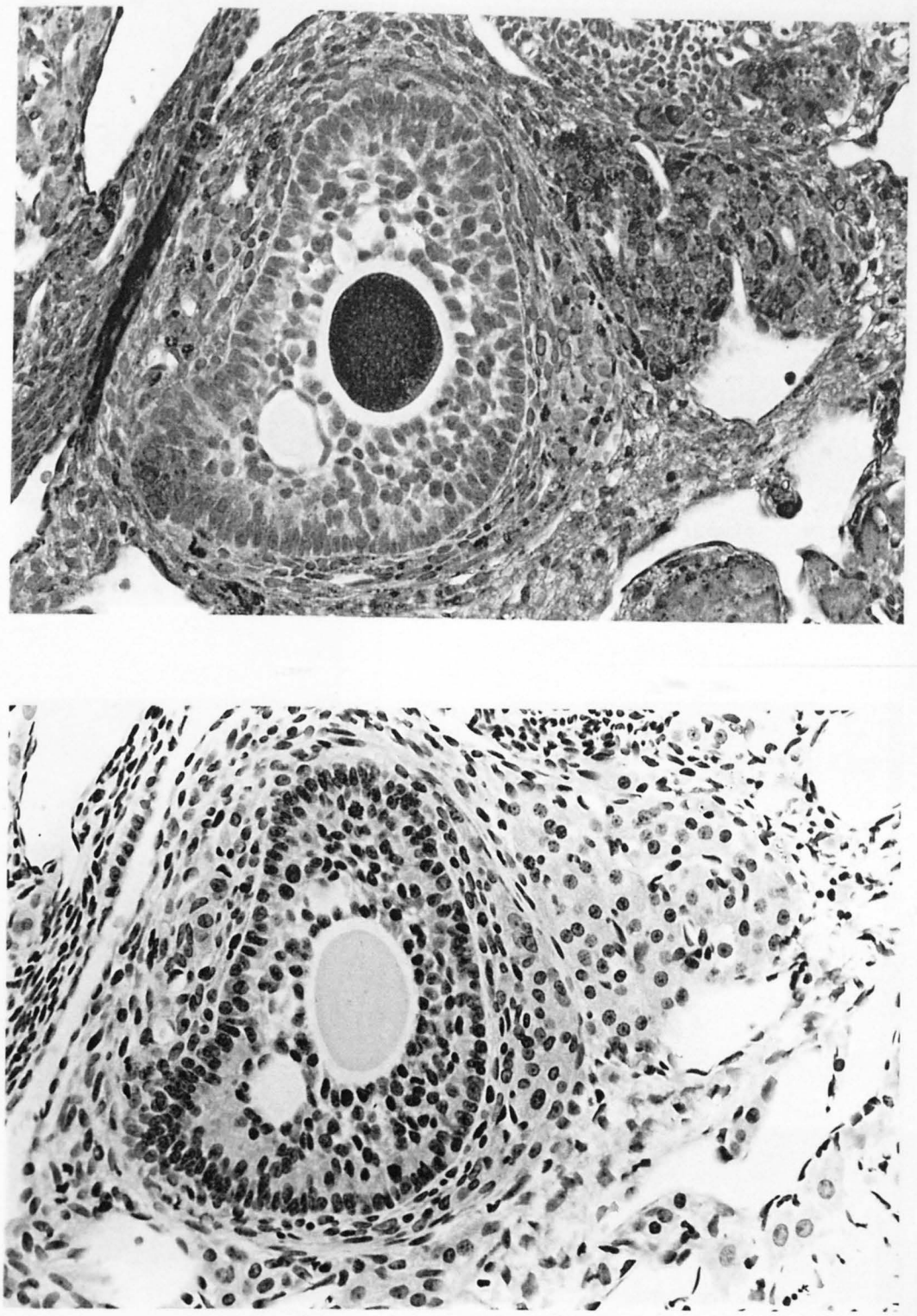


Figure 3.4 Immunohistochemistry of a Rat Ovary

The top shows immunostaining of rat ovary using antiserum to purified rat liver 11ß-hydroxysteroid dehydrogenase, displaying marked staining of the oocyte. Bottom is control using pre-immune serum, showing no immunostaining. Magnification is 400 times.

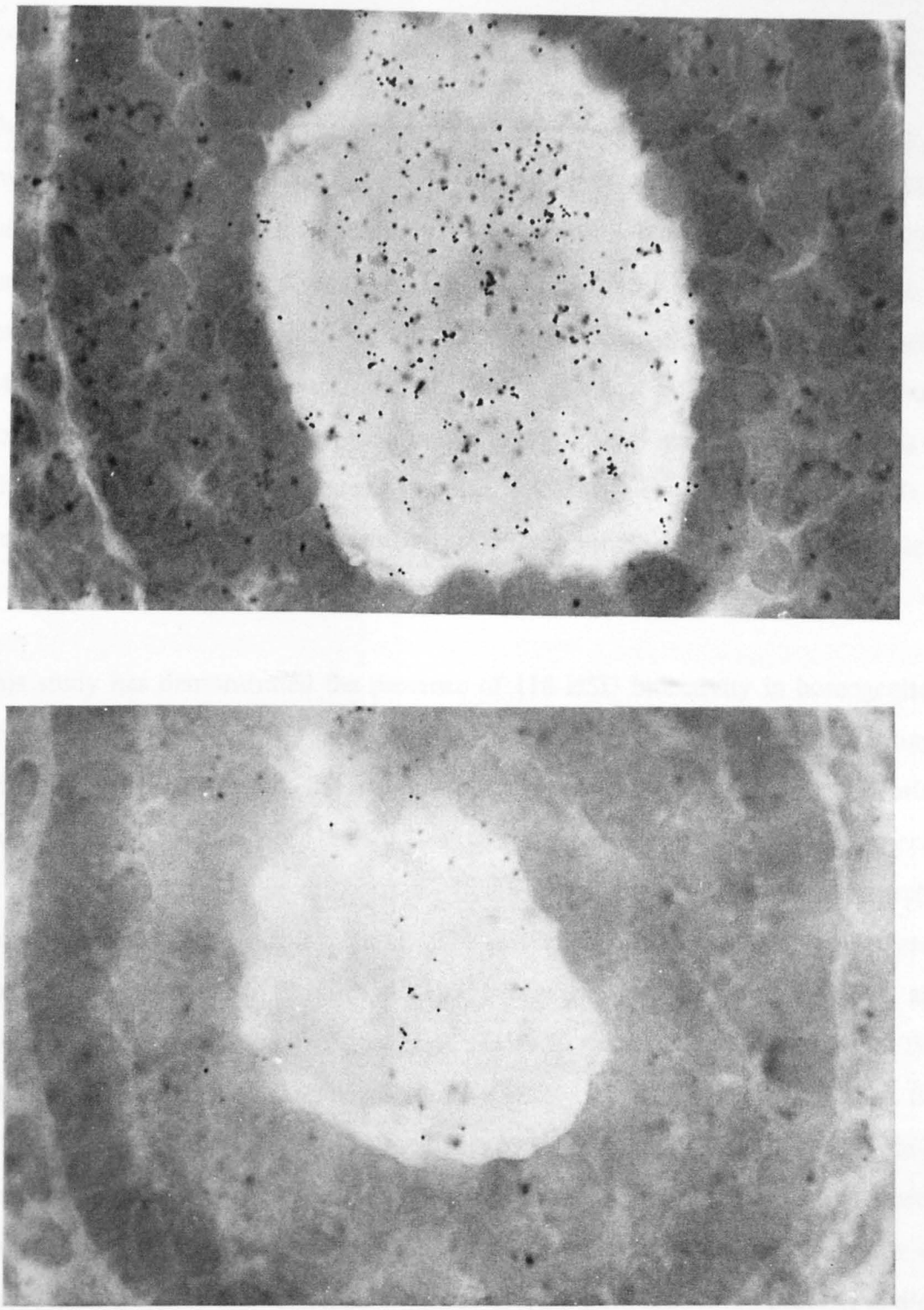


Figure 3.5 Rat ovary In-Situ Hybridisation

Top is *in-situ* hybridisation showing a concentration of silver grains over the oocyte indicating hybridisation of the cRNA probe containing ^{35}S-labelled UTP to 11ß-HSD mRNA. The bottom part is *in-situ* hybridisation using "sense" probe, showing no silver grain concentration. Magnification: x600.

3.4. DISCUSSION

The purpose of the studies reported in this Section, was first and foremost to try to answer the question whether 11ß-HSD was present in the ovary, a question which has been surrounded with controversy. Thus in 1975, Ghraf [Ghraf et al., 1975] and colleagues reported absence of 11ß-HSD bioactivity in the rat ovary but in contrast, Murphy, in 1981 found considerable bioactivity in human ovaries [Murphy 1981]. Furthermore, glucocorticoids do affect gonadal function in both sexes and 11ß-HSD has been found in male rat gonads [Phillips et al., 1989] as in other glucocorticoid target tissues. One would therefore expect to find 11ß-HSD in the ovary where, by analogy with the testis (see introduction, page 88) it might have a developmental role.

This study has demonstrated the presence of 11ß-HSD bioactivity in homogenised whole ovaries using NAD as cofactor, in agreement with Murphy's findings [Murphy 1981]. Murphy did not however use cofactors, and in adult ovaries found equal activities for both 11-oxidation and 11-reduction, whereas only oxidation was detected in this study. In foetal and childhood gonads, Murphy found only oxidation [Murphy 1981]. The discrepancy between the data presented here and those of Ghraf [Ghraf et al., 1975] (who used both NAD and NADP), are unclear but may relate to the very high pH (9.5) used in the latter study. The activity at identical protein concentrations is only 10% of the kidney activity, the kidney being a tissue known to have abundant 11ß-HSD. This could be due to: i) sparse/scanty cellular expression of the enzyme in the ovaries (as has been demonstrated here - see below), and/or ii) a narrow (perhaps cyclical) window of expression, possibly related to the oestrous cycle (or follicle development), and/or iii) the presence of inhibitors of the enzyme in the ovary. The last possibility is in fact supported by Figure 3.3, where the reaction appears inhibited at protein concentrations beyond 0.5 mg/ml (only looking at incubation time of 10 min, with 200 μM NAD and 12 nM ^{3}H-corticosterone). There are of course a number of candidate steroids that might act as inhibitors in the ovary, including progesterone,

as reviewed in detailed by Monder & White [Monder & White 1993]. The findings reported here have since been confirmed, i.e. 11ß-HSD bioactivity is present in cultured ovarian cells, more specifically, in cultured granulosa lutein cells, where the direct inhibition of LH-induced steroidogenesis by glucocorticoids is modified by 11ß-HSD [Michael et al., 1993b].

Using both immunohistochemistry and *in-situ* hybridisation, liver-type 11ß-HSD (the antibodies and cDNA derived from liver-type enzyme) was localised to the oocytes themselves, and to a lesser extent to luteal bodies. Thus 11ß-HSD is not ubiquitous, many components of the ovary not expressing the enzyme. The northern blot indicates only one species of this liver type enzyme, in agreement with the data of Tannin [Tannin et al., 1991], and as observed in hippocampus, cerebellum and testis [Moisan et al., 1990a; Moisan et al., 1990b], which contrasts with multiple hybridising forms found in the kidney, both in this study and others [Krozowski et al., 1990; Moisan et al., 1992b]. These multiple forms are due to differential promoter usage of the 11ß-HSD-1 gene [Moisan et al., 1992a], which when expressed *in-vitro* are inactive [Mercer et al., 1993; Obeid et al., 1993]. The possibility of multiple forms (derived from different genes rather than variants of 11ß-HSD-1), is also apparent in male rat reproductive organs where bioactivity is found in both testis and epididymis but immunoreactivity to liver-derived 11ß-HSD is only observed in testis [Monder 1991].

Data from our laboratory [Brown et al., 1993] has shown that the liver type (11ß-HSD-1) is exclusively NADP dependent and reflects a separate gene product from placental (and renal) 11ß-HSD-2, which is NAD dependent (see also report of cloning of 11ß-HSD-2 [Albiston et al., 1994]). The bioactivity reported in this study was NAD dependent (NADP was not looked at except in isolated oocytes - see results page 92), and recent reports [Piercy et al., 1993] point to the bioactivity in human cultured granulosa-lutein cells also being NAD dependent. However, in contrast to the NAD dependent bioactivity, liver-type 11ß-HSD immunoreactivity and mRNA expression was found here, - localised to the oocyte and luteal bodies. It is of course not possible to localise at a cellular level the NAD dependent

bioactivity in crude ovarian homogenates but this raises several possibilities. Firstly the NAD supported bioactivity might be explained by a variant of 11ß-HSD-1 which immunoreacts with the antibody, but can use NAD, although this is unlikely as no such activity has been found for any of the variant transcripts of the 11ß-HSD-1 gene [Mercer et al., 1993; Obeid et al., 1993]). Secondly the enzyme might be an unknown variant of 11ß-HSD-2 which is recognised by the antibody against liver-type 11ß-HSD. As 11ß-HSD-2 is thought to be an almost exclusive dehydrogenase [Brown et al., 1993; Albiston et al., 1994], the lack of reductase activity here supports this, but against it is the observation that purified placental 11ß-HSD-2 is not immunoprecipitated by 11ß-HSD-1 antisera [Brown et al., 1993]. The third possibility, namely the two isoforms co-existing is also not unlikely, each playing separate roles - as possibly is the case in the baboon placenta where direction of reaction seems to change as pregnancy advances (see later chapters). The fourth possibility is of course that this polyclonal liver-derived antibody is not detecting authentic 11ß-HSD, but simply cross-reacting with something else, a notion supported by the presence of cross reacting species on western blots [Phillips et al., 1989]. Finally, this "something else" could of course be a third as yet not characterised isoform of 11ß-HSD (i.e. product of a third gene).

This study found a specific pattern of 11ß-HSD mRNA expression, like in the testis where 11ß-HSD is found specifically in the Leydig cells of the testis-proper (as opposed to for example the epidydymis). Perhaps surprisingly, no indication of the presence of 11ß-HSD in granulosa cells was found, although these cells have previously been shown to contain glucocorticoid receptors [Schreiber et al., 1982] and to be metabolically regulated by glucocorticoids [Hsueh & Erickson 1978; Adashi et al., 1981; Wang & Leung 1989]. Also, this study failed to demonstrate the presence of 11ß-HSD in theca cells, although the synthetic glucocorticoid dexamethasone has been shown to alter their content of the enzyme carbonyl reductase [Inazu et al., 1990]. This does however not preclude the presence of 11ß-HSD-2 in these locations. The finding of 11ß-HSD immunoreactivity and

mRNA expression in the luteal cells, but not the theca cells, may be another example of cellular ontogeny, as we have previously shown in the skin [Teelucksingh et al., 1990], where 11ß-HSD is not detected in the basal cell layer, but is located in the upper epidermal cells. The ontogeny/differentiation idea has been supported by Michael and colleagues who have subsequently [Michael et al., 1993b] found bioactivity in cultured granulosa cells that have undergone luteinisation.

The specific pattern of localisation and the presence of NAD dependent bioactivity is very interesting and throws open the question of what the role of ovarian 11ß-HSD might be. The proposed physiological function of 11ß-HSD-1 is activation of inactive steroids (reductase activity), in the liver explaining why oral cortisone is active as a glucocorticoid, a notion which has been supported recently by data from our laboratory where the protein encoded by a liver derived cDNA functions as a reductase in intact mammalian cells [Low et al., 1994]. The liver-type enzyme may however be bi-directional as suggested by findings in the testis, where 11ß-HSD-1 immunoreactivity is found [Phillips et al., 1989], and bioactivity is predictably NADP dependent [Campbell et al., 1992] but the role appears to be to inactivate steroids (11ß-dehydrogenation) [Monder et al., 1994a; Monder et al., 1994b].

Could the role of ovarian 11ß-HSD be to increase availability of glucocorticoids to the maturing oocyte? In the context of our knowledge of the deleterious effects of glucocorticoids on growth *in-utero* (see introduction to thesis), what beneficial effects could possibly be derived from that? A well known example of a beneficial effect of glucocorticoids is term foetal lung maturation/surfactant production, which is induced by glucocorticoids [Hundertmark et al., 1994]. For the oocyte, suppression of local maternal inflammatory/immunological responses to this half foreign body may be beneficial at implantation, and induction of oocyte enzymes necessary for development could also be important.

There is some recent data supporting this idea that glucocorticoids may be necessary for very early development. Thus a recent publication [Michael et al., 1993a] found that the absence of 11ß-HSD in cultured human granulosa cells aspirated from subjects undergoing *in-vitro* fertilisation-embryo transfer (IVF-ET), predicted implantation success i.e. pregnancy. The authors felt that this implied that lack of glucocorticoid inactivation by 11ß-HSD was beneficial. It is however hard to see how lack of 11ß-HSD activity in cultured granulosa lutein cells can predict successful implantation, the cells having been cultured for 3 days (luteinised) before 11ß-HSD activity was measured. Another problem is the classical understanding that steroidogenesis by the luteal bodies is necessary for maintenance of pregnancy. This steroidogenesis is inhibited by glucocorticoids, and the inhibition is alleviated by 11ß-HSD [Michael et al., 1993b]. The absence of 11ß-HSD would therefore lead to greater inhibition of steroidogenesis by glucocorticoids. It is of course possible that one or both of the 11ß-HSD isoforms in the ovary have nothing to do with glucocorticoid metabolism, and that the substrate is a different steroid(s).

In summary, the original quest for the presence of 11ß-HSD in the ovary has been successful. Subsequently, others have published very interesting data highlighting the potential role of 11ß-HSD as a modulator of development before implantation. This opens up numerous avenues which would be exciting to explore. The immediate next step would of course be to do *in-situ* hybridisation and northern blotting using the probes against NAD dependent placental 11ß-HSD-2, which only just now is becoming available.

4. RAT PLACENTAL 11ß-HSD

4.1. INTRODUCTION

Recent epidemiological studies show that low birth weight is associated with death from ischaemic heart disease [Barker et al., 1989a], and increased risk of developing several risk factors for ischaemic heart disease, including high blood pressure. The association with higher blood pressures holds in children [Barker et al., 1989a; Law et al., 1991; Cater & Gill 1984], adolescents [Seidman et al., 1991], and adults [Barker et al., 1989a; Barker et al., 1990; Gennser et al., 1988]. Some of the studies have found that the unusual combination of low birth weight with a large placenta, is strongly associated with later hypertension [Barker et al., 1990]. The pathogenesis is obscure but has been attributed to maternal malnutrition [Barker et al., 1993a]. However, this combination of low birth weight and high placental weight has also been observed in rats with streptozotocin-induced diabetes mellitus [Robinson et al., 1988; Canavan & Goldspink 1988]. These animals have increased maternal glucocorticoid levels [Heller et al., 1988]. Previous work has shown that exogenous glucocorticoids retard foetal growth in both humans and animals [Reinisch et al., 1978; Benediktsson et al., 1993; Katz et al., 1990] and that exogenous glucocorticoids given to pregnant rats can increase placental weight, possibly depending on timing and dose [Gunberg 1957]. We treated pregnant rats with the synthetic glucocorticoid dexamethasone [Benediktsson et al., 1993]. The offspring were lighter than controls at birth, and although reared normally (as were controls) from birth, developed elevated blood pressure 5 months later. Thus, was it possible that glucocorticoid excess *in-utero* might be responsible for imprinting (programming) of cardiovascular disease? What is the barrier that normally protects the foetus

from the much higher maternal glucocorticoid levels? Could there be an intrinsic variation in the effectiveness of this barrier?

Several authors have proposed that this placental glucocorticoid barrier is the enzyme 11ß-hydroxysteroid dehydrogenase [Murphy et al., 1974; Dancis et al., 1978; Beitins et al., 1973; Dormer & France 1973] which catalyses the conversion of the metabolically active cortisol (11ß-hydroxysteroid), to the inactive cortisone (11-oxosteroid). Although cultured fibroblasts from human uterine tissue had been shown to be able to catalyse 11ß-ol oxidation in 1958 [Sweat et al., 1958], it was not until in 1960 that bioactivity studies by Osinski [Osinski 1960] demonstrated the presence of 11ß-HSD in term human placenta.

Several other investigators have since confirmed this in humans and in various other species, although controversy has surrounded several aspects of the story (see Section 1.4.5 for a detailed account). In humans, 85% of maternally injected radiolabelled cortisol at 13 - 18 weeks of pregnancy, was converted to cortisone on transplacental transfer [Murphy et al., 1974]. Deduced from similar human experiments at term, only 25% of term foetal cortisol is derived from the mother, while 90% of foetal cortisone is of maternal origin [Beitins et al., 1973]. Using *ex-vivo* perfusion of intact human placentas at term, Dancis found that most of maternal cortisol was converted to inactive cortisone during passage across the placenta [Dancis et al., 1978]. Several *in-vitro* studies have supported this, López Bernal's group suggesting that human placental 11ß-HSD was an effective barrier to maternal glucocorticoids throughout gestation [López Bernal & Craft 1981; Blasco et al., 1986]. There seem however to be important inter-species differences. Thus in the baboon, 11ß-HSD at mid-gestation appears to catalyse cortisone to cortisol conversion, while at term the reverse (dehydrogenation, inactivating cortisol) [Pepe & Albrecht 1984b]. It appears that in the baboon,

oestrogens which induce cortisol to cortisone conversion, are responsible for this change in enzyme direction [Pepe et al., 1988].

In order to test the hypothesis that i) a considerable natural variation exists in placental 11ß-HSD activity (this variation presumably resulting in variable foetal glucocorticoid exposure), and ii) that this variation, if found, correlates with foetal growth, a study of 11ß-HSD activity in normal Wistar rats was undertaken. Rats were chosen as an experimental model for initial exploration of this hypothesis since i) the *in-utero* dexamethasone programmed hypertension model was the rat, ii) the rat placental anatomy with regard to materno-foetal exchange resembles closely human anatomy (Section 1.5), iii) a cDNA for, and antibody to rat liver 11ß-HSD-1 was available, and iv) the species is easy to mate, handle and was readily available.

4.2. METHODS

The methodology of the 11ß-HSD activity assay is described in detail in Section 2.4.1. In brief, on day 20 of pregnancy (term is 20 - 22 days), rats were killed by cervical dislocation. The placentas and foetuses were immediately placed on ice and weighed. The placentas were homogenised in KRB and the protein content of the homogenate estimated according to the method of Bradford [Bradford 1976]. The enzyme assay was carried out in duplicate incubations at 37 °C. Substrate was ^{3}H-corticosterone and NAD was cofactor, as for all assays, it was made up fresh in KRB before each assay and stored on ice, protected from light. Total incubation volume was 250 μl and incubation of buffer alone provided an assay blank. The incubation was started by adding tritiated steroid and terminated by adding 2.5 ml of ethyl acetate, which also served to extract the steroids before separation with

automated HPLC. Enzyme activity was expressed as % conversion ^{3}H-corticosterone to ^{3}H-11-dehydrocorticosterone.

Animals were lethally perfused with paraformaldehyde and placentas post fixed for immunohistochemistry. Detection was by the peroxidase-antiperoxidase method [Sternberger et al., 1970], using an antibody against rat liver 11ß-HSD raised in rabbits, kindly donated by Dr. C. Monder [Monder & Lakshmi 1990]. Preimmune rabbit serum served as control. For *in-situ* hybridisation, fresh placentas were immediately at surgery frozen on dry ice. Cryostat sections were mounted onto slides and post fixed for *in-situ* hybridisation. An antisense cRNA probe was transcribed from a vector containing 11ß-HSD cDNA insert [Agarwal et al., 1989] and hybridised to the sections. Controls were hybridised with similarly labelled (^{35}S-UTP) non-complementary "sense" RNA probes under identical conditions.

4.3. RESULTS

4.3.1. Bioassay Evaluation

As the literature on cofactor preference of placental 11ß-HSD was discrepant (see Section 1.4.5), NAD/NADP preference was examined. As shown in Figure 4.1, the rat placental enzyme activity is highly inducible with NAD, the enzyme showing clear preference of NAD over NADP at 200 μM and 2000 μM. There did not seem much additional advantage of using higher concentration than 200 μM NAD which was therefore used for all subsequent assays.

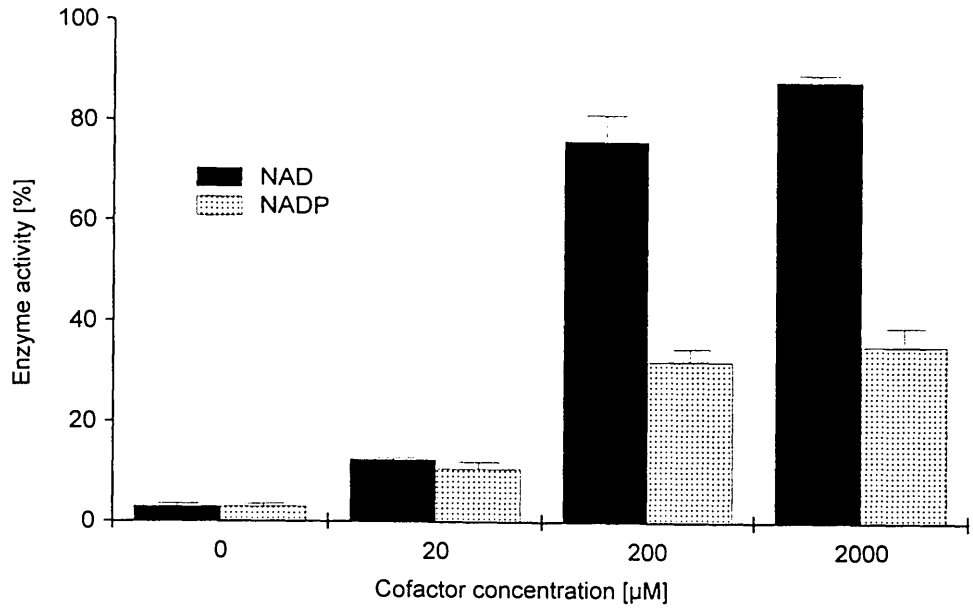


Figure 4.1 Cofactor Preference of Rat Placental 11ß-HSD *In-Vitro*

Enzyme activity of 11ß-HSD expressed as % conversion B to A without cofactor added and at three different final concentrations of NAD and NADP. Each column represent mean + SEM for 3 placentas incubated in duplicate. Protein content was 0.5 mg/ml, ^{3}H-corticosterone was 12 nM final, incubated for 60 min.

The next step was plotting the progress of reaction as a function of enzyme concentration (protein concentration) and time. The left hand panel in Figure 4.2 shows that the progress of the reaction slows down beyond protein concentration of 0.2 mg/ml. The progress of reaction is linear with respect to enzyme added beyond 0.2 mg/ml. The progress of reaction with increased incubation time and protein concentration of 0.5 mg/ml is shown in the right hand panel of Figure 4.2. Linearity was observed up to approximately 20 minutes.

Although blank rates were always negligible and identical to blanks using denatured homogenate, it was still important to show that the conversion was due to 11ß-HSD, and thus could be inhibited with classic 11ß-HSD inhibitors. The active constituent of liquorice, glycyrrhetinic acid, and the synthetic hemisuccinate of glycyrrhetinic acid, the water soluble ulcer healing drug carbenoxolone were used. The results are shown in Figure 4.3. Approximate IC_{50} for both glycyrrhetinic acid and carbenoxolone was very similar, being 0.9×10^{-5} M and 1×10^{-5} M respectively.

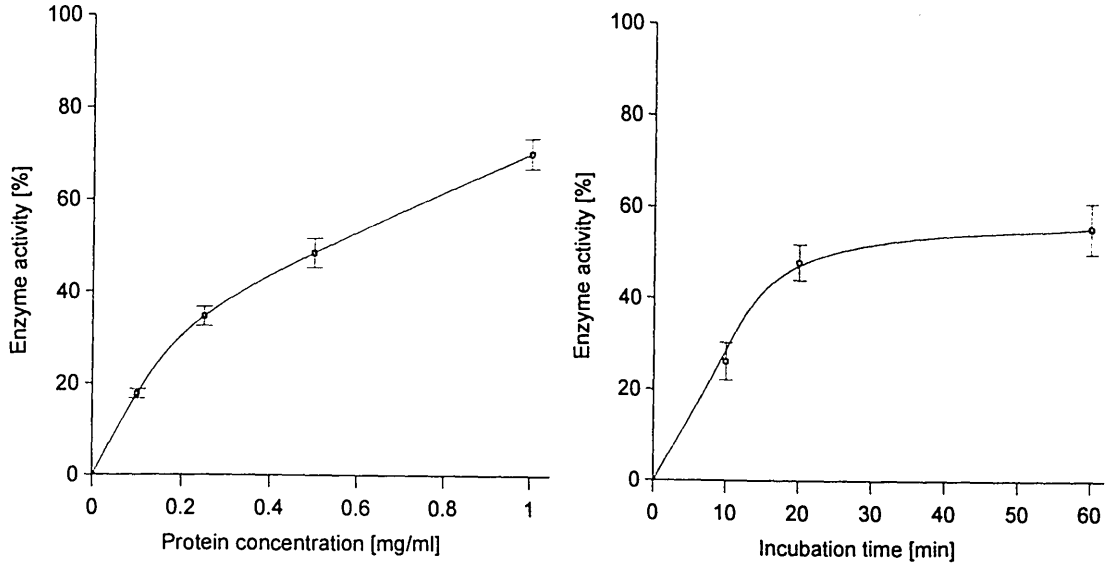


Figure 4.2 Rat Placental 11ß-HSD *In-Vitro* Assay

The vertical axis indicates % conversion corticosterone (B) to 11-dehydrocorticosterone (A). On the left panel the progress of reaction is in two phases, linearity with respect of protein added being observed beyond protein concentration of ca. 0.2 mg/ml. On the right hand panel the progress of reaction is linear for the first 20 min using 0.5 mg/ml protein. Each point represents mean of 4 placentas incubated in duplicate. Bars indicate ± SEM. Cofactor was NAD 200 µM final, substrate ^{3}H-corticosterone at 12 nM final.

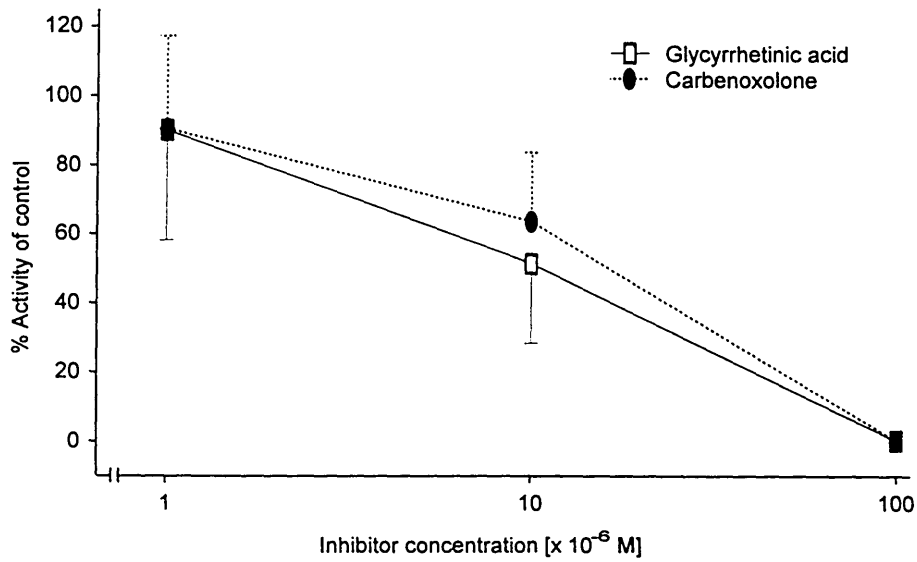


Figure 4.3 Rat Placental 11ß-HSD Inhibition *In-Vitro*

The effect of three concentrations of carbenoxolone and glycyrrhetinic acid on the conversion of ^{3}H-corticosterone to ^{3}H-11-dehydrocorticosterone. The data is activity in the presence of inhibitor as a percentage of control activity set at 100% (in the absence of inhibitor). Points represent mean of 3 - 5 experiments, while bars indicate ± SEM. The horizontal axis is logarithmic.

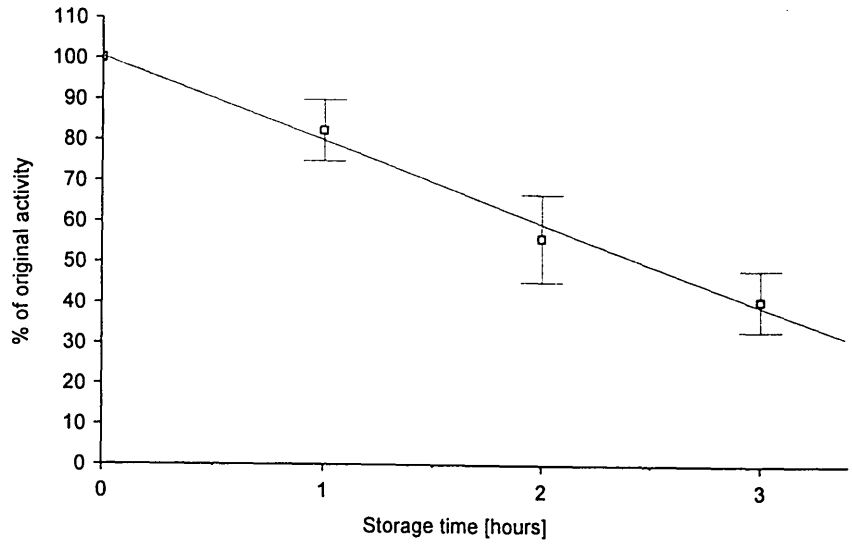


Figure 4.4 Storage of Rat Placental Homogenate

The figure shows a linear decline in homogenate 11ß-HSD bioactivity when stored on ice. Time zero is first incubation, 1h after homogenisation. Storage time therefore represents additional hours. Data is expressed as % of original activity for 3 different placentas, each incubated in duplicate. Points represent mean and bars ± SEM.

Several reports in the literature comment on the instability of 11ß-HSD with storage although some researchers have not noted that. In order to examine that possibility, two placentas were cut in half, one half assayed fresh and the other half immediately frozen at -70° C. The following day the frozen halves were thawed, homogenised and assayed as usual. The enzyme proved very unstable, the mean activity for the two placentas (each incubated in duplicate) being 81%, dropping down to 18% on freezing, i.e. maintaining only 22% of the original activity. But was this enzyme only unstable on freezing? Looking at the stability of the homogenate, kept immersed in ice, a linear decline in activity with time was found (Figure 4.4), activity declining by approximately 15% per hour. Thus timing had to be very precise, keeping the time from homogenisation to start of incubation fixed. The time from killing of the animal to start of homogenisation was constant (30 min) and similarly the time from homogenisation to start of incubation (60 min). The recovery of radioactive label was 71% and CV = 5% (n = 7). Intra-assay coefficient of variation was 3% at approximately 50% conversion (n = 11).

The inter-assay variation could not of course be assessed due to the instability of the placental enzyme.

4.3.2. Relationship of 11ß-HSD to Rat Foetal Growth

In order to determine whether rat placental 11β-HSD was capable of excluding active maternal glucocorticoid from the foetal circulation, relative dehydrogenase (inactivating glucocorticoids) and reductase (generating active glucocorticoid) activities were looked at. The substrate for reductase was ^{3}H-11-dehydrocorticosterone, using NADH as cofactor at 200 μM. At 3 stages of gestation, early (12 d; n = 2), mid (16 d; n = 4) and term (20 d; n = 3), no reductase activity was found and the dehydrogenase activity was similar at all stages.

As discussed in the introduction to this thesis, the rat placenta consists of two zones, the labyrinthine zone where the materno-foetal exchange takes place, and the basal zone. Although maternal blood has to travel through the basal zone, localisation in the labyrinthine zone would probably be most efficient for 11ß-HSD to function as a barrier. The data presented so far was derived from whole homogenised placentas. Demonstrating that bioactivity was specifically present in the labyrinthine zone was thus important. A natural plane of cleavage exists between the basal and labyrinthine zones, pulling on the basal zone with pliers easily peeled it off the labyrinthine zone. Each part was homogenised separately as previously described. The result is shown in Figure 4.5. 11ß-HSD bioactivity is present in both parts, under the conditions studied the activity in the labyrinthine zone was slightly, but not significantly, higher than the activity in the basal zone.

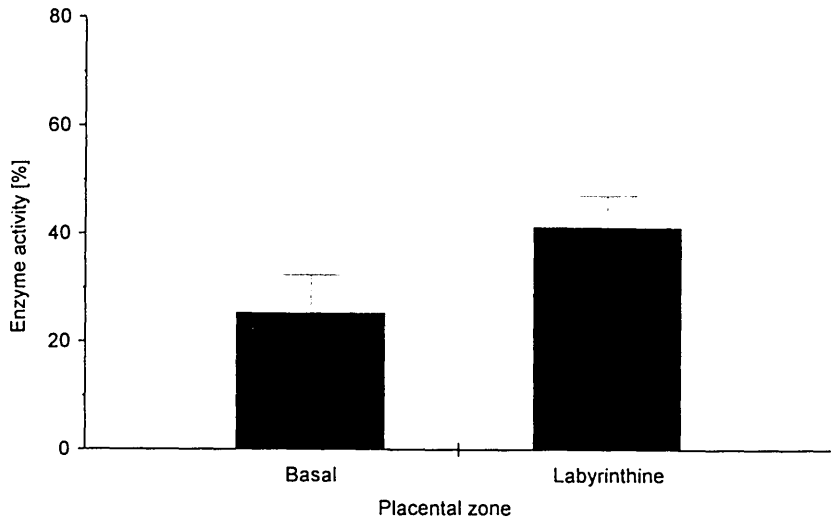


Figure 4.5 Zonal Distribution of 11ß-HSD Bioactivity

Although the bioactivity is slightly higher in the labyrinthine zone, this does not reach statistical significance using t-test. Data are mean ± SEM, n = 4; triplicate incubations.

Could there be a physiological variation in this barrier (which seems to be predominantly oxidative throughout pregnancy)? This question was addressed by estimating placental enzyme activity in 56 placentas from 8 normal Wistar rats at term. A strong, positive ($r = 0.46$, $r^2 = 0.21$), highly significant ($p < 0.0005$) correlation was found between placental 11ß-HSD activity and term foetal weight (Figure 4.6). This indicates that those foetuses that were smallest at term had the lowest placental 11ß-HSD activity and were thus presumably exposed to the greatest amount of maternal glucocorticoids. Processing the data as means per litter showed the same, albeit stronger, positive correlation ($r = 0.96$, $r^2 = 0.92$, $p < 0.001$; $n = 8$). An apparent outlier with regard to foetal weight is indicated with an arrow in Figure 4.6. Excluding this outlier from the analysis gives $r = 0.65$, $r^2 = 0.42$ and $p < 0.0000001$.

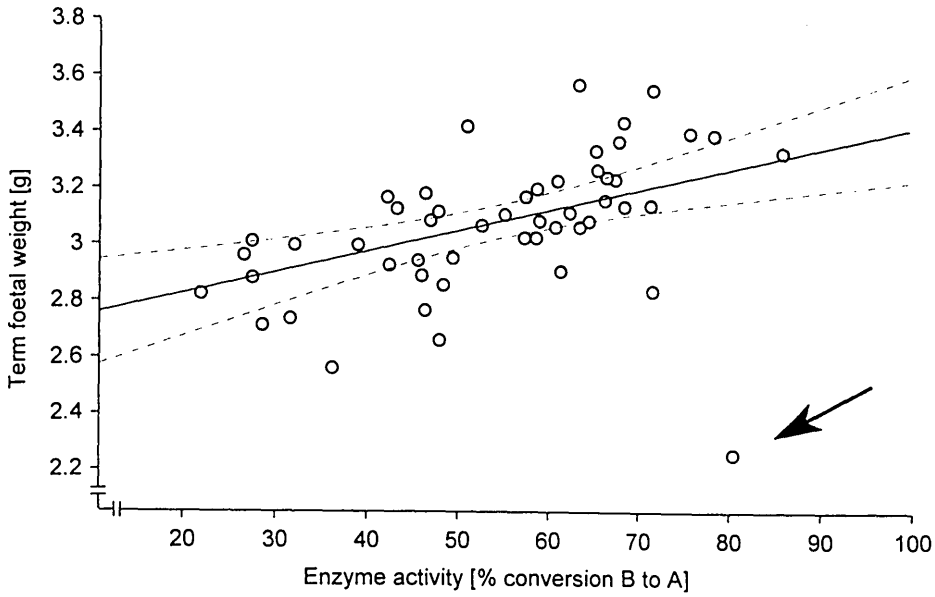


Figure 4.6 Term Rat Foetal Weight and 11ß-HSD Bioactivity

A highly significant positive correlation of term placental enzyme activity (x-axis) with term foetal weight (abscissa). Pearson's r is + 0.46, r^2 = 0.21, $p < 0.0005$, n = 56. The solid line is the regression line and dotted lines indicate the 95% confidence interval for the regression. Note an outlier (arrow), which is included in data analysis.

Again using Pearson's product moment correlation, but this time for the relationship between term placental weight and term placental 11ß-HSD activity (Figure 4.7), a strong negative ($r = -0.63$, $r^2 = 0.40$), highly significant ($p < 0.00001$) correlation was found. Similar results were obtained when data were analysed as means per litter ($r = -0.66$, $p < 0.05$, $n = 8$). There was no apparent placental weight outlier. Thus the biggest placentas have lowest 11ß-HSD activity, on a per unit weight basis.

The question at this point was of course (with reference to the human data on the combination of low birth weight and high placental weight predicting high blood pressure): What is the form of the relationship between placental 11ß-HSD activity at term and both foetal- and placental weights simultaneously? In other words, do small rat foetuses which also have a large placenta, have low placental 11ß-HSD activity, and those that are large with small placentas high placental 11ß-HSD activity. This is explored in Figure 4.8, where the data has been plotted in 3 dimensions, enzyme activity on the vertical axis while placental and foetal weights

are displayed on the two horizontal axes. What is evident is the general trend for small foetuses with large placentas to have lower placental 11ß-HSD activity than big foetuses with small placentas, although the relationship seems to be less clear at the extreme low placental weights and the extreme high foetal weight.

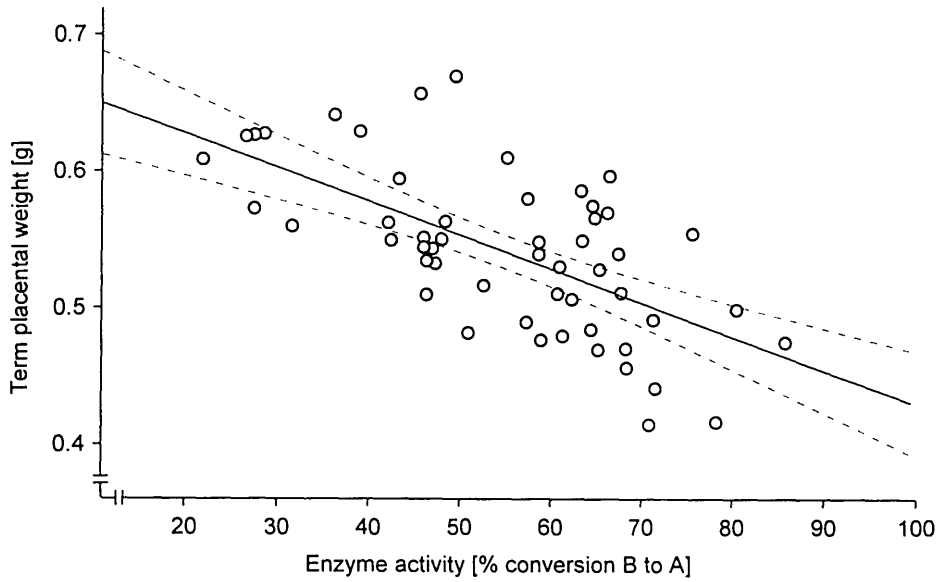


Figure 4.7 Term Rat Placental Weight and 11ß-HSD Bioactivity

A highly significant negative correlation of term placental enzyme activity (x-axis) with term placental weight (abscissa). Pearson's r is -0.63, $r^2 = 0.40$, $p < 0.00001$, $n = 56$. The solid line is the regression line and dotted lines indicate the 95% confidence interval for the regression.

It is possibly more relevant with regard to foetal glucocorticoid exposure, to look at some measure of total placental capacity to metabolise glucocorticoids. A way of doing that would be to multiply the % conversion ^{3}H-corticosterone to ^{3}H-11-dehydrocorticosterone per g of placental homogenate by the total placental weight (see discussion for criticism). Using this approach, a strong positive correlation of term foetal weight and "total placental 11ß-HSD activity" was again found ($r = 0.45$, $r^2 = 0.20$, $p < 0.0005$). It is of course meaningless to correlate total placental 11ß-HSD activity with placental weight as placental weight then contributes substantially to both sides of the equation.

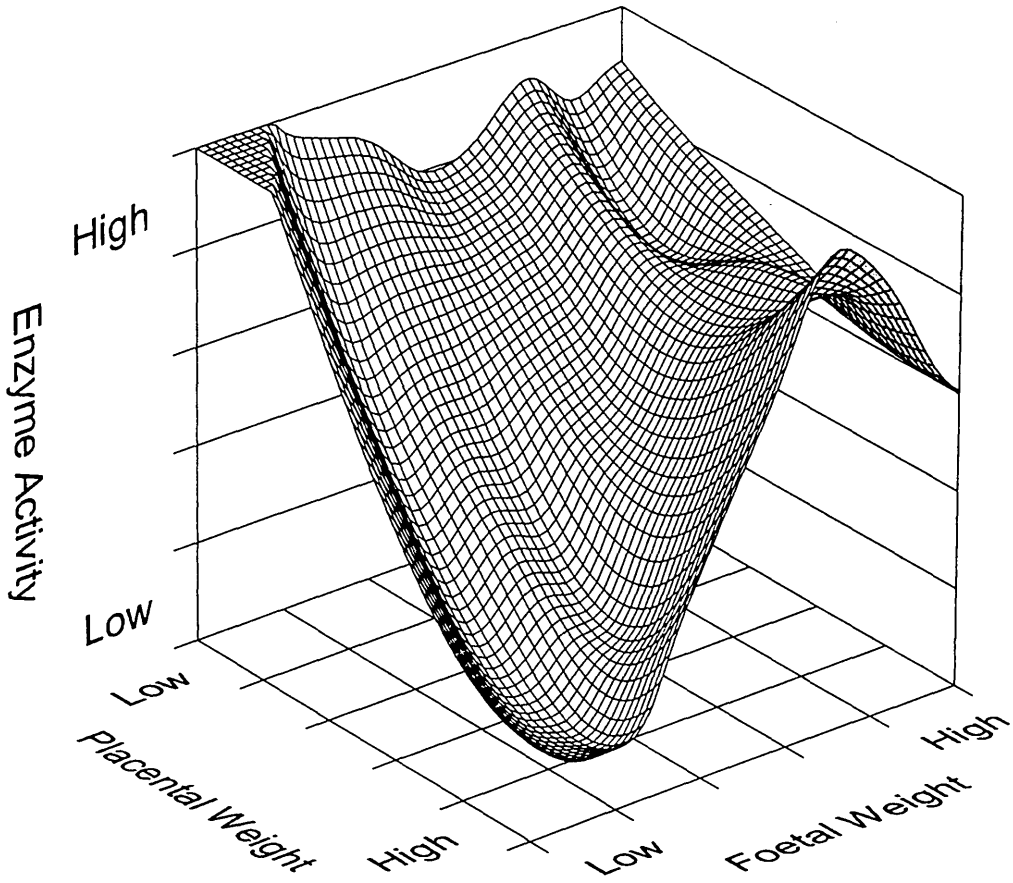


Figure 4.8 Rat Placental 11ß-HSD Activity, Term Foetal and Placental Weight

The data points (not shown) have been fitted with a contour map employing a "least squares" method. It is important to appreciate that this figure can only indicate trends as the actual data points do not reach the corners of the figure.

4.3.3. Immunohistochemistry and mRNA expression

Using the same antibody as was used for ovarian immunohistochemistry (donated by Dr. C. Monder; raised in rabbits to rat liver 11ß-HSD), a strong positive continuous band of staining was found in the barrier separating the maternal and foetal circulations in the labyrinthine zone of the placenta (Figure 4.9). Additionally, there was immunostaining of moderate intensity the vacuolated glycogen cells [Davies & Glasser 1968], of the basal zone. The *in-situ* hybridisation pattern paralleled the immunohistochemistry findings, being positive in the barrier between the two circulations and over the vacuolated glycogen cells (Figure 4.10).

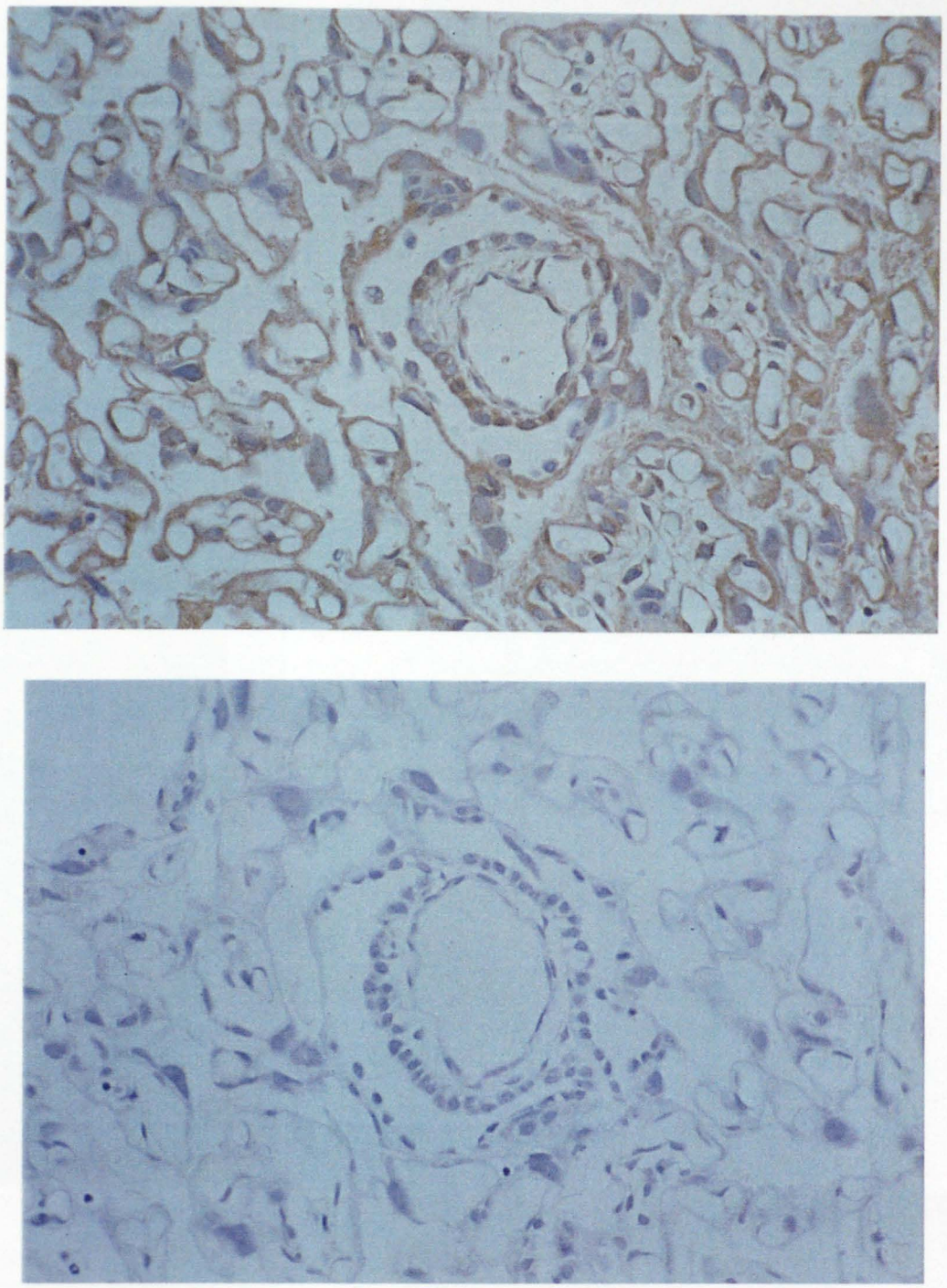


Figure 4.9 Immunohistochemistry of Term Rat Placenta

The figure shows a portion of rat placental labyrinthine zone. The top shows positive immunoreactivity in the barrier separating the maternal and foetal circulations (brown colour), indicating presence of 11ß-HSD-1 protein. Bottom is control using pre-immune serum, showing no staining.

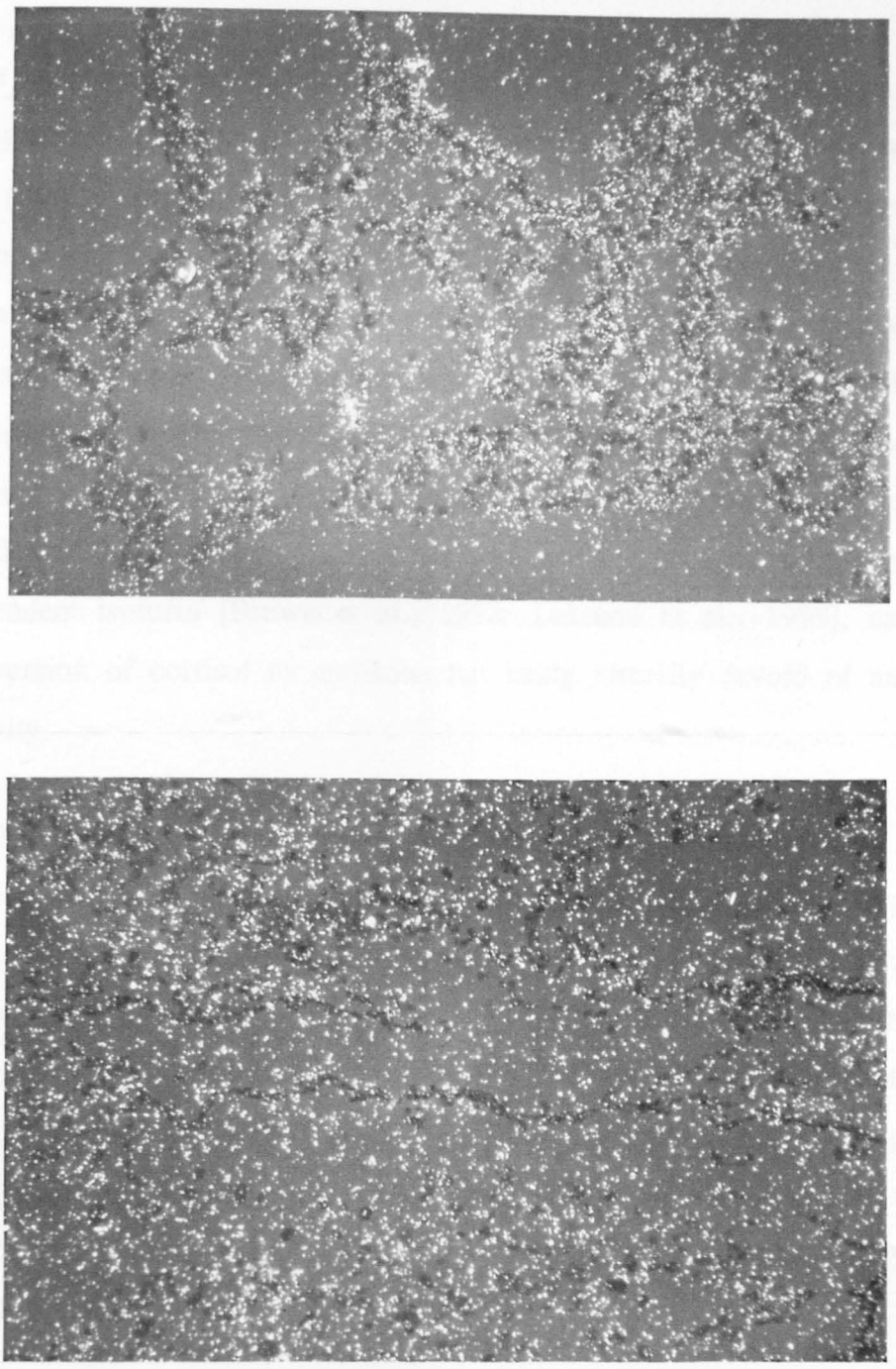


Figure 4.10 Term Rat Placenta In-Situ Hybridisation

The figure shows a portion of rat placental labyrinthine zone. The top panel is *in-situ* hybridisation showing a concentration of silver grains over the barrier between the maternal and foetal circulations, indicating hybridisation of the cRNA probe containing ^{35}S-labelled UTP to 11ß-HSD mRNA. In-situ hybridisation using "sense"-probe, showing uniform silver grain distribution.

4.4. DISCUSSION

This study has found abundant NAD dependent 11ß-HSD dehydrogenase activity in rat placentas throughout pregnancy, which was present in both the basal and labyrinthine zones - the labyrinthine zone being where the materno-foetal exchange takes place. Before this study was performed there was considerable discrepancy apparent in the literature regarding cofactor preference of placental 11ß-HSD. Thus some found NADP preference [Blasco et al., 1986; Osinski 1960; López Bernal et al., 1980b] some NAD [Pepe & Albrecht 1984a] and some no preference [Meigs & Engel 1961]. More recently, the findings presented here have been confirmed, as the human placenta has been found to predominantly express a NAD dependent isoform [Brown et al., 1993; Lakshmi et al., 1993], catalysing the conversion of cortisol to cortisone but being virtually devoid of any reductase activity.

Furthermore, it has a K_m in the low nanomolar range, thus probably being better suited to function as a barrier to maternal glucocorticoids than liver-type 11ß-HSD (11ß-HSD-1) which has a K_m 100 times higher (rat liver) [Brown et al., 1993], and which probably functions physiologically to convert cortisone to cortisol (or 11-dehydrocorticosterone to corticosterone, i.e. reductase) [Duperrex et al., 1993; Low et al., 1994; Moore et al., 1993]. This "placental isoform" is a product of a gene distinct from the gene for 11ß-HSD-1 [Brown et al., 1994], and is very similar to (one amino acid difference) the recently cloned human kidney 11ß-HSD-2 [Albiston et al., 1994].

It is of course entirely plausible that there is more than one isoform of 11ß-HSD present in the placenta, as suggested by López Bernal [López Bernal et al., 1980b] and Lakshmi [Lakshmi et al., 1993]. Although in the studies presented here, no reductase activity could be found (early-, mid-, late-gestation), others have found minimal amounts of reductase [Giannopoulos et al., 1982; Lakshmi et al., 1993; Brown et al., 1993], but the dehydrogenase activity was always predominant. The

reductase seems to be more fragile [Brown et al., 1993; Lakshmi & Monder 1985; Lakshmi et al., 1993], and thus could have been destroyed here during tissue processing. Thus, in spite of the lack of reductase bioactivity, it is not possible on the basis of the findings here to exclude the presence of 11ß-HSD-1. Similarly, if both isoforms are active in the preparation, but dehydrogenation is dominant, it would be impossible to detect any activity attributable to 11ß-HSD-1 (reduction), as the product (corticosterone) would immediately be converted back to 11-dehydrocorticosterone.

The data presented do in fact support the presence of 11ß-HSD-1 as well as NAD dependent 11ß-HSD-2. First, some NADP dependent dehydrogenase activity was demonstrated although NAD dependent activity was predominant. NADP dependent placental activity has also been found by others (see above), and has been attributed to a different (from the NAD dependent form), but co-existing isoform, as judged by analysis of Eadie-Hofstee plots [Brown et al., 1993]. Other tissues, for example testis Leydig cells also express 11ß-HSD-1 [Phillips et al., 1989], but show NADP dependent dehydrogenation of active corticosterone to inactive 11-dehydrocorticosterone [Monder et al., 1994a; Monder et al., 1994b]. The second line of evidence for the presence of 11ß-HSD-1, arising from the data presented in this thesis is the presence in the rat placenta of i) immunoreactivity using an antibody raised against 11ß-HSD-1 (rat liver), and ii) the expression of 11ß-HSD-1 mRNA. The presence of protein and/or mRNA is not the equivalent of bioactivity - the protein could be inactive.

Not only is the reductase known to be unstable in the placenta, but in this study the NAD dependent dehydrogenase was also found to be fragile (Figure 4.4). The explanations for this could for example be the presence of proteases in the homogenate, inappropriate pH (although pH 7.4 may be physiological, that may not promote stability in homogenates), mechanical disruption etc.. This fragility would serve to dilute any relationship between foetal growth and placental 11ß-HSD activity and thus importantly, the time from tissue harvesting until assay was kept constant.

The progress of reaction slowed down beyond approximately 0.2 mg/ml, although it was linear with respect of amount of enzyme added. This could be due to the presence of an endogenous reversible inhibitor in the homogenate. Dozens of steroids have been shown to reversibly inhibit 11ß-HSD [Monder & White 1993], but those most likely to be the culprits in this tissue are pregnenolone or rather progesterone [Pepe & Albrecht 1984a], while other steroids of placental origin like 17-hydroxyprogesterone are not likely [Pepe & Albrecht 1984a]. Oestrogen probably induces placental 11ß-HSD, at least in the baboon [Baggia et al., 1990b]. Accumulation of product (11-dehydrocorticosterone), is of course another possibility.

The IC_{50} for the placental enzyme was here found to be approximately 10^{-5} M for both carbenoxolone and glycyrrhetinic acid. This is in keeping with previously published values for human placenta [Teelucksingh et al., 1991a], and values presented in Section 6.3.2. The homogenate used here was rather coarse, making the presence of some intact cells likely. The IC_{50} for intact cells in other tissues expressing 11ß-HSD-2 [Monder et al., 1989], or in crude homogenates of tissues expressing 11ß-HSD-1 [Walker 1992] is also comparable to the values obtained here, whereas in purified tissue preparations (microcosms etc.), IC_{50} seems to be several orders of magnitude lower (10^{-7} - 10^{-9} M) [Monder et al., 1989; Schleimer 1991], which is probably explained by better access of the inhibitors to the enzyme.

The anatomical localisation of bioactivity to the labyrinthine zone (in addition to the basal zone), where the materno-foetal exchange takes place, is compatible with the proposed role of placental 11ß-HSD, that is, to be a barrier to maternal glucocorticoids. However, conclusive proof for 11ß-HSD being the barrier can only be obtained by inhibiting 11ß-HSD in intact perfused placentas, demonstrating abolition of the barrier. Supportive evidence can though be claimed for the studies presented here, where glucocorticoid metabolism was completely inhibited with the classical 11ß-HSD inhibitors, glycyrrhetinic acid and carbenoxolone. The considerable variation in activity of 11ß-HSD (22 - 86% conversion of ^{3}H-

corticosterone to ^{3}H-11-dehydrocorticosterone) found here is also compatible with the hypothesis that different foetuses are likely to be exposed to variable amounts of maternal glucocorticoids with potential adverse effects.

The highly significant direct correlation with term foetal weight, of the ability of placental 11ß-HSD to inactivate corticosterone ($r = 0.46$, $p < 0.0005$), observed in this study, is supportive of the notion that placental 11ß-HSD plays a crucial role in ensuring low foetal glucocorticoid levels, allowing normal development of the foetus. It is well established that exogenous glucocorticoids do retard foetal growth in both humans and animals [Reinisch et al., 1978; Katz et al., 1990], and key in this context, are our studies on *in-utero* induced hypertension by treatment of pregnant rats with the synthetic glucocorticoid dexamethasone (which is incompletely metabolised by 11ß-HSD) [Benediktsson et al., 1993]. Further studies in the rat have also shown that treatment of pregnant animals with carbenoxolone results in low birth weight, and hypertensive offspring [Lindsay et al., 1994b], actions which are dependent on the presence of intact maternal adrenal glands [Lindsay & Seckl 1994].

How glucocorticoids might programme adult hypertension is obscure but perinatal programming (imprinting) is well recognised for glucocorticoids and other steroid hormones. The potential mechanisms are discussed in detail in 1.1.3. But what about the malnutrition hypothesis? Malnutrition is a stress, and thus leads to increased glucocorticoid levels, but additionally, protein malnutrition in pregnant rats (which also leads to offspring hypertension [Langley & Jackson 1994]) has recently been shown to potently depress placental 11ß-HSD activity [Phillips et al., 1994], potentially marrying the two hypotheses.

The findings here that firstly, the placental enzyme activity on a per gram basis is lowest in the heaviest placentas ($r = -0.63$, $p < 0.00001$), and secondly, that these heaviest placentas correspond to the lightest foetuses, are intriguing. These animals are likely to be exposed to the greatest amount of maternal glucocorticoid. Supporting the view that this is not a spurious relationship, are the epidemiological

data on the prediction of higher blood pressure levels by the combination of low birth weight and high placental weight [Barker et al., 1990], exactly the pattern found here.

Not all the epidemiological studies have found this, and the mechanism of disproportional placental enlargement remains unexplained; rather than being a direct glucocorticoid effect on the placenta it could be secondary (or compensatory) to a failure of foetal growth as observed in hypoxia and anaemia [Alexander 1978; Godfrey et al., 1991]. The work of Robinson, Owens and De Barro [Robinson et al., 1994] might also provide an explanation. They have shown in sheep, that the response of the placenta to malnutrition at mid-pregnancy is critically dependent on the nutritional state of the mother at conception. Compared to well fed ewes (at mating and throughout pregnancy), the foetal/placental weight ratio was lower (relative placental enlargement) if the ewes were light (malnourished) at mating, with the ratio even lower if those malnourished ewes received unrestricted nutrition during mid-pregnancy.

The foetus itself has also been shown to independently regulate placental growth and placental IGF-2 production [Putney et al., 1991]. This pattern of low birth weight and high placental weight, is found in offspring of rats made diabetic with streptozotocin [Robinson et al., 1988; Canavan & Goldspink 1988], an experimental model where maternal glucocorticoid levels are markedly raised [Heller et al., 1988]. Interestingly, this model has elevated foetal expression of IGF binding proteins 1 and 2 [Ooi et al., 1990], but glucocorticoids potently regulate IGF-2, the type 1 IGF receptor and binding proteins 1 and 2 [Li et al., 1993; Price et al., 1992].

The findings discussed in the previous paragraphs, are based on enzyme activity per gram of placental homogenate. This standardisation allows comparison of different placentas. A weakness is of course that the placenta does contain a considerable amount of blood, which will be present in the homogenate, and thus contribute to its protein content. It is also possible that what is important for the

foetus, is not the enzyme activity per gram of placenta, but rather, total placental capacity to metabolise glucocorticoids. Multiplying the activity per g by the total placental weight, again showed a significant direct correlation with term foetal weight ($r = 0.45$, $p < 0.0005$).

There are however problems with this approach. Thus (apart from the question of blood content raised above), there might be areas in the placenta, which do not contain any enzyme activity and therefore total wet placental weight might be misleading. Of course no association can be sought, using this approach, for placental weight and total placental activity as placental weight is a major component on both sides of the equation. The only way of sorting this out conclusively would be to do perfusions of intact placentas, preferably *in-vivo*, and at different stages of gestation. That would also bypass any potential problems with isoform contributions, cofactor preferences and the predominant direction of the reaction.

What then is the role of 11ß-HSD-1 in the placenta? Although the precise role of the vacuolated glycogen cells in the basal zone of the rat placenta (where 11ß-HSD-1 protein and mRNA is present - bioactivity was also found in the basal zone) is not clear, the presence of glycogen and the co-localisation of 11ß-HSD-1 protein and mRNA to these cells, proposes a role for 11ß-HSD-1 in placental energy metabolism. Another potential role is in placental growth (see above), since insulin-like growth factor 2 (IGF-2), which has been shown to be important in placental growth [Barker et al., 1993a; Li et al., 1993], is regulated by glucocorticoids [Price et al., 1992]. Also, a modulatory role in placental steroidogenesis is conceivable by analogy to the testis and ovary [Michael et al., 1993b; Monder et al., 1994a].

As regards the labyrinthine zone, where both 11ß-HSD-1 protein and mRNA are present in the barrier between the maternal and foetal circulations, the potential role is intriguing. Does it function as a reductase as has been proposed elsewhere [Duperrex et al., 1993; Low et al., 1994; Moore et al., 1993]? Are

glucocorticoids needed for foetal growth? Or does it operate there as a dehydrogenase, as it appears to do in the testis [Monder et al., 1994a; Monder et al., 1994b]? Some evidence for a placental reductase is available from studies in the baboon, where overall placental 11ß-HSD activity seems to favour generation of active glucocorticoids at mid-gestation, but at term the reverse [Pepe & Albrecht 1984b]. This seems to be regulated by oestrogens, and may serve to regulate the development of the foetal hypothalamic-pituitary-adrenal axis [Pepe et al., 1988].

In summary, some evidence has been presented here implicating a role for placental 11ß-HSD in the development of adult disease, particularly hypertension. There are however some problems, and it was felt important to look at this question in humans, where the epidemiological observations had been made. Thus a study of human placental 11ß-HSD was undertaken, although some of the problems encountered here with homogenates would be likely to crop up again. Thus only looking at intact fresh human placenta would be likely to provide conclusive answers.

5. HUMAN PLACENTAL 11ß-HSD IN-VITRO

5.1. INTRODUCTION

In humans, low birth weight, sometimes in association with high placental weight, predicts higher blood pressures in later life. This is observed already at a few weeks of age and the association grows stronger with the age of the population studied [Law et al., 1993]. We put forward the hypothesis that this association might be due to relative glucocorticoid excess *in-utero*, caused by a dysfunction of the presumptive placental glucocorticoid barrier, 11ß-HSD [Benediktsson et al., 1993; Edwards et al., 1993a].

In support of this hypothesis we demonstrated in the rat (Section 4) a strong direct association between placental 11ß-HSD activity *in-vitro* and term foetal weight ($r = 0.42$; $p < 0.0005$) and a negative association with term placental weight ($r = -0.63$; $p < 0.00001$). The foetuses presumably exposed to the greatest amount of maternal glucocorticoids (low placental 11ß-HSD activity) were not only smallest but also had the largest placentas, which is the pattern predicting higher human blood pressures in some studies (not all epidemiological studies have demonstrated the inverse relationship between adult blood pressure and placental weight; a mechanism explaining this variation is lacking). In addition we could induce offspring hypertension in rats by treating the pregnant mothers with dexamethasone [Benediktsson et al., 1993], and moreover, treatment of pregnant rats with inhibitors of 11ß-HSD (carbenoxolone) also resulted in low weight offspring which went on to develop adult hypertension [Lindsay et al., 1994b; Lindsay et al., 1994a; Lindsay & Seckl 1994].

At least two isoforms of 11ß-HSD exist. 11ß-HSD-1, initially purified from liver, is a reversible, NADP dependent enzyme that may predominantly reactivate inert cortisone (or 11-dehydrocorticosterone) [Duperrex et al., 1993; Low et al., 1994;

Moore et al., 1993]. In contrast, the human placenta expresses a distinct high affinity predominantly NAD dependent [Brown et al., 1993] isoform, 11ß-HSD-2 (the rat placenta also expresses predominantly this isoform: Section 4.3.1), that appears from *in-vitro* studies, to have biochemical properties appropriate to allow effective metabolism of the maternal cortisol load [Brown et al., 1993], protecting the human foetus from maternal cortisol throughout pregnancy [Murphy et al., 1974; Beitins et al., 1973; Pasqualini et al., 1970; Blasco et al., 1986]. However, this barrier appears not to be complete, some maternal cortisol reaching the foetus, and the relationship if any between placental 11ß-HSD efficiency *in-vivo* and foetal growth in man is unknown.

It was of course important to try and determine if placental 11ß-HSD played a similar role in humans as it appeared to do in the rat. In order to address this it was necessary to examine whether human placental 11ß-HSD activity was related to foetal and placental growth, and to look at the relationship between human placental 11ß-HSD activity and markers of foetal glucocorticoid exposure. It was also important to determine whether any other enzymes metabolised cortisol in the human placenta, (i.e. whether 11ß-HSD was in fact the placental glucocorticoid barrier) and whether the correlation with birth weight (if found) was a general phenomenon, observed with any enzyme, or specific to 11ß-HSD.

There was a catch however; 11ß-hydroxysteroid dehydrogenase activity in either homogenised or whole rat placentas is very unstable *in-vitro* (Figure 4.4). This instability has also been observed for human placental homogenates [Brown et al., 1993; Lakshmi et al., 1993]. Any *in-vitro* data (in homogenates) has therefore to be interpreted with care, and it would be of key importance to supplement any *in-vitro* enzyme activity studies with data obtained using different methodologies more closely related to the *in-vivo* function of the intact placenta. One such method, which is thought to closely mimic the *in-vivo* situation, is the "*ex-vivo* human placental cotyledon perfusion" of freshly isolated intact placentas. This approach was therefore developed (see Section 6). Section 7 deals with *in-vivo* markers of glucocorticoid exposure and human placental 11ß-HSD. Nevertheless, the

remainder of this Section reports data from the initial studies which estimated 11ß-HSD activity in homogenates of human placenta, using the same methodology as used in the rat. In an attempt to see if any correlation with birth weight was a general phenomenon (not specific for 11ß-HSD), 2 other enzymes known to be stable on storage (Dr. T. Bramley) were assayed, namely alkaline phosphatase (ALP) and N-γ-L glutamyl ß-naphthylaminidase (GNA), both membrane markers, unrelated to steroid or fuel metabolism.

5.2. METHODS

5.2.1. Subject Selection and Sample Collection

Validation experiments were carried out on freshly isolated human placentas obtained from the Simpson Memorial Maternity Pavilion in Edinburgh. Immediately following delivery the placenta was put in a plastic bag, cooled on ice and used as soon as possible. For correlation of enzyme activity with birth weight we had the opportunity to take part in a prospective study in collaboration with Professor Barker's group in Southampton. This was an ongoing study which involved collecting placental samples from 528 consecutive singleton deliveries. At delivery, placental and birth weights were measured in a standardised manner. Trophoblast specimens (1 -2 g) were taken from a healthy looking part of each placenta and immediately frozen and stored at -70 °C. These samples were then used to study in-vitro 11ß-HSD, ALP and GNA activities in trophoblast homogenates.

5.2.2. Assays

Assay methodology is described in detail in Section 2.4. In brief, trophoblast samples were homogenised in KRB, the protein content of the homogenate estimated according to the method of Bradford [Bradford 1976], and a portion of the homogenate immediately frozen at -70°C for assay of ALP and GNA activities.

The 11ß-HSD enzyme assays were carried out in duplicate incubations at 37 °C. In preliminary validation studies, assay conditions were defined. Optimal conditions were defined as being on the linear part of the "rate of reaction curve" for time, cofactor and protein concentrations (see below). The total incubation volume was 250 μl and incubation of buffer alone provided an assay blank. As an internal standard to allow inter-assay coefficient of variation (CV) to be calculated, individually frozen samples from the same fresh placenta were defrosted on the day of each assay. The incubation was started by adding tritiated steroid (12 nM 3H-cortisol) and terminated by adding 2.5 ml of ethyl acetate, which also served to extract the steroids before separation with automated HPLC (Section 2.4).

The assays for ALP and GNA were validated and carried out by Dr. T. Bramley at the Centre for Reproductive Biology, Edinburgh. These were both photometric assays (described in Section 2.4), both enzymes are stable on storage at -70 °C.

5.3. RESULTS

5.3.1. 11ß-HSD Bioassay Evaluation

There was a clear cofactor preference in the rat placental 11ß-HSD assay, the enzyme favouring NAD as shown in Figure 4.1. In order to ascertain the preference of human placenta using this methodology, fresh human placental homogenate was incubated in the presence of variable concentrations of NAD and NADP. As in the rat, there was a preference for NAD (Figure 5.1).

Evaluation studies for fresh human placental 11ß-HSD activity are shown in Figure 5.2, where cofactor concentration is 40 μM and ^{3}H-cortisol was 12 nM. The progress of reaction at 0.2 mg/ml was linear for up to about 15 min, as shown in the left hand panel. The right hand panel plots the effect of increasing protein concentration for an incubation time of 15 min. The reaction progress is linear for protein concentration up to 0.4 mg/ml. The conditions chosen for future assays were 40 μM NAD, 12 nM ^{3}H-cortisol, 0.2 mg/ml protein, incubated for 10 min.

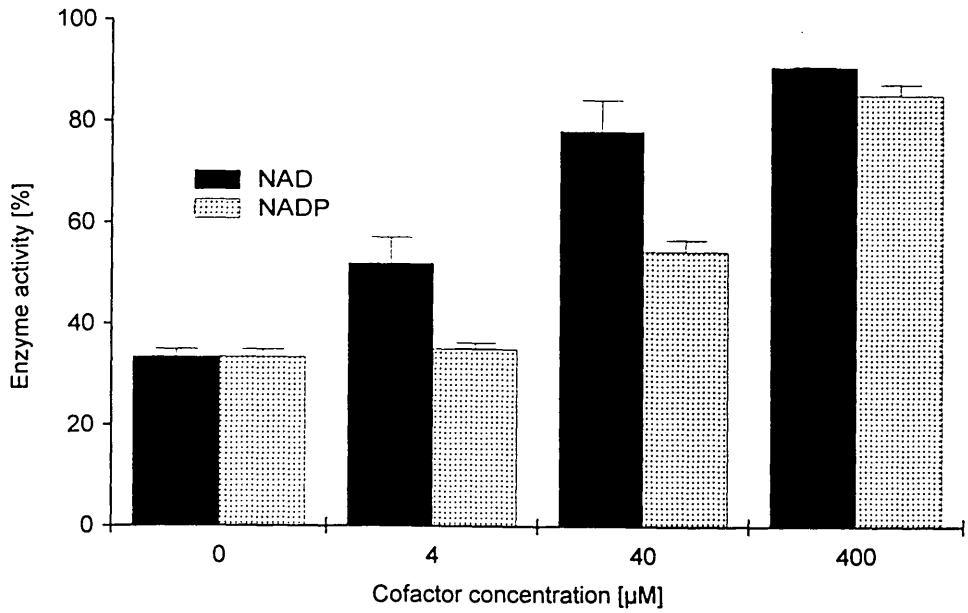


Figure 5.1 Cofactor Preference of Human Placental 11ß-HSD *In-Vitro*

Enzyme activity of 11ß-HSD expressed as % conversion F to E without cofactor added and at three different final concentrations of NAD and NADP. Each column represents the mean of 3 experiments. Bars indicate SEM. Placental samples were incubated in duplicate. Protein content was 0.5 mg/ml, ^{3}H-cortisol was 12 nM final, incubated for 60 min at protein concentration of 0.5 mg/ml.

As the samples were kept frozen, it was important to show that the enzyme activity could be preserved on storage and that the enzyme behaved similarly in thawed specimens and fresh. Thus before embarking upon the collaborative study on birth weight, 4 placentas were assayed fresh and several samples from those placentas (whole trophoblast) then individually frozen. These specimens were thawed, homogenised and assayed with the same conditions as when fresh on a number of occasions. As shown in Figure 5.3, the activity was apparently stable for up to 35 days ($p = 0.68$ on ANOVA). The frozen-thawed placentas were then taken through the same series of experiments as presented in Figure 5.2, in order to check whether the assay conditions were still appropriate. These data are presented in Figure 5.4, which shows that the previously chosen assay conditions still are on the linear part, the curves on both panels showing remarkable similarity. The same assay conditions were thus chosen for determining 11ß-HSD activity in both fresh and frozen-thawed trophoblast specimens, including the study on birth weight in collaboration with Professor Barker's group.

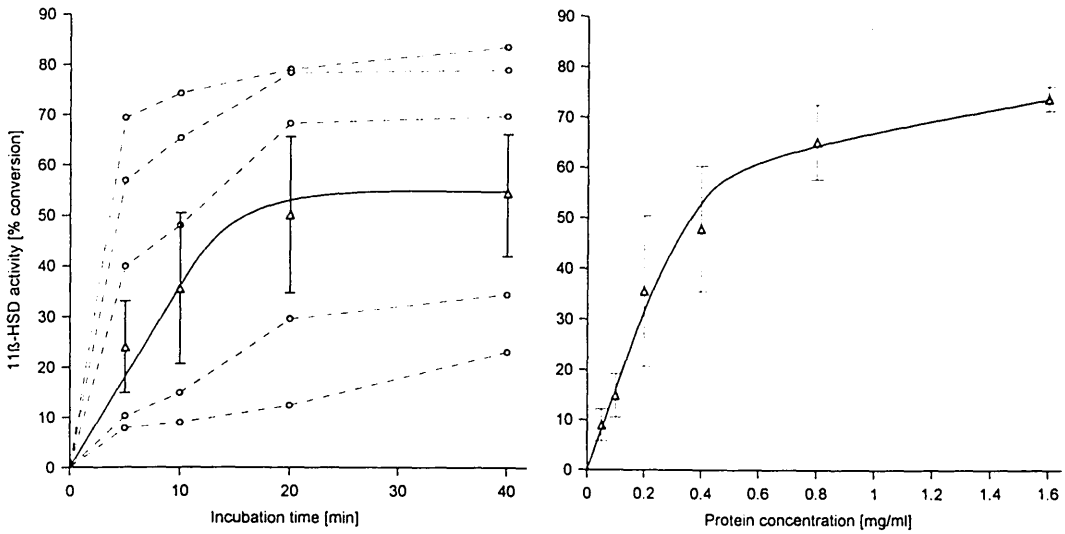


Figure 5.2 Human Placental 11ß-HSD *In-Vitro* Assay: Fresh Trophoblast

Each curve in the left hand panel indicates progress of reaction with time at different protein concentrations. From top 1.6 mg/ml, 0.8 mg/ml, 0.4 mg/ml, 0.2 mg/ml (solid line with error bars), 0.1 mg/ml, 0.05 mg/ml. For clarity, error bars are omitted from and dotted lines indicate each protein concentration except for 0.2 mg/ml. The right hand panel shows progress of reaction at 10 min for different protein concentrations. Each point is n = 3, error bars indicate mean ± SEM. ^{3}H-cortisol was 12 nM final and NAD was 40 µM final.

Recovery of label was 91%, CV was 14% (n = 10). Intra-assay CV was 3% (fresh placenta, n = 10) and inter-assay CV 18% (frozen trophoblast samples, n = 31). Only one product was detected, i.e. the only two peaks on the HPLC were cortisone and cortisol. The question of inhibition with known inhibitors of 11ß-HSD (glycyrrhetinic acid and/or carbenoxolone) had been addressed for the rat in Section 4.3.1, and for humans it was more important to assess the effect of those inhibitors on the enzyme in the *ex-vivo* perfusion (Section 6.3.2). No reductase activity (conversion of 11-dehydrocorticosterone to corticosterone) was detected with 40 μM NADH and 12 nM ^{3}H-11-dehydrocorticosterone.

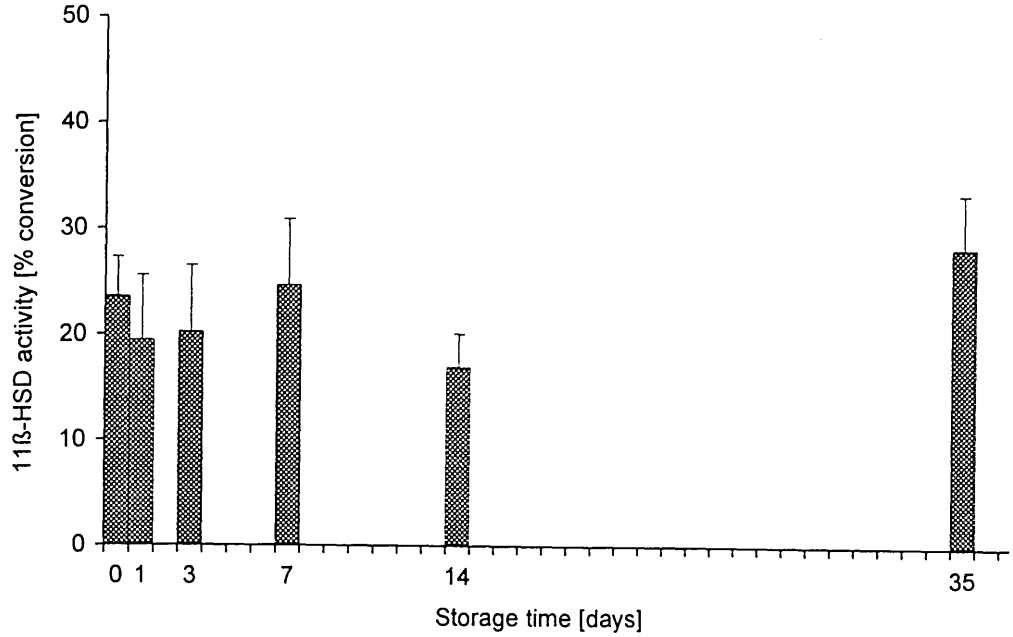


Figure 5.3 Short Term Storage of Human Placental 11ß-HSD

Storage at time = 0 (the column farthest to left) is 11ß-HSD activity in fresh placentas. The activity remained unchanged for up to 35 days (as judged by ANOVA, p = 0.68) compared to fresh activity. Columns indicate mean of 4 experiments (placentas) while bars indicate SEM.

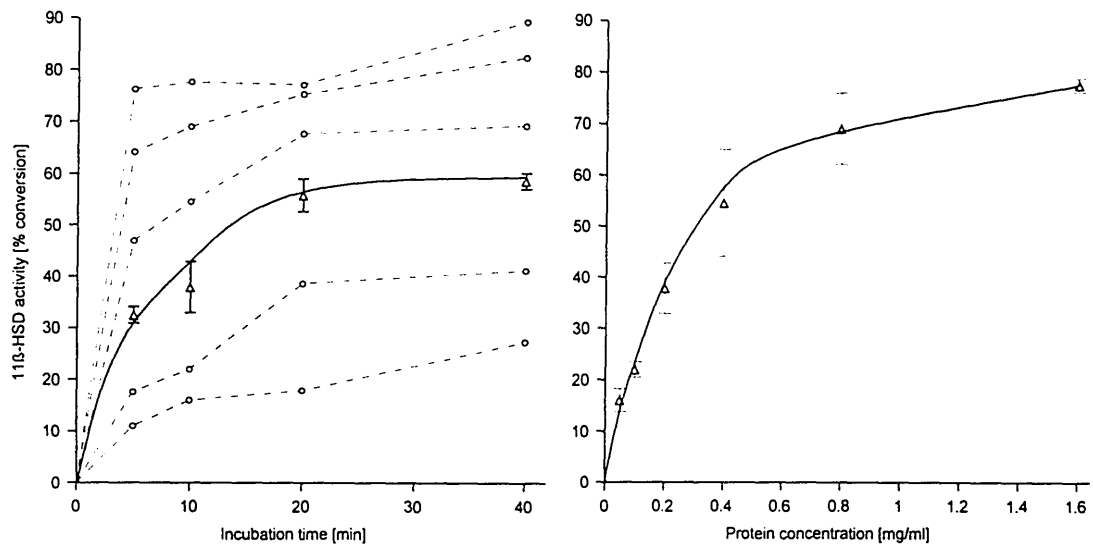


Figure 5.4 Human Placental 11ß-HSD *In-Vitro* Assay: Stored Trophoblast

Each curve in the left hand panel indicates progress of reaction with time at different protein concentrations. From top 1.6 mg/ml, 0.8 mg/ml, 0.4 mg/ml, 0.2 mg/ml (solid line with error bars), 0.1 mg/ml, 0.05 mg/ml. For clarity, error bars are omitted from and dotted lines indicate each protein concentration except for 0.2 mg/ml. The right hand panel shows progress of reaction at 10 min for different protein concentrations. Each point is n = 3, error bars indicate mean ± SEM. ^{3}H-cortisol was 12 nM final and NAD was 40 µM final.

5.3.2. Birth Weight and Human 11ß-HSD In-Vitro

Overall details of the study subjects are shown in Table 5.1. Note that the full data set was not available for all 528 enrolled subjects. 522 had placental samples taken. The spread of both birth weights and placental weights is very large. Birth weight was significantly correlated with length of gestation ($r = 0.56$, $p << 10^{-6}$, $n = 524$), and with placental weight ($r = 0.60$, $p << 10^{-6}$). The activity of 11ß-HSD was expressed in the conventional way as % conversion of substrate to product ($[^{E}/_{F+E}] \times 100$). The activities of ALP and GNA were expressed as nmoles/min/mg protein. Mean 11ß-HSD activity was 16.6% under the conditions used, and showed considerable variation (SD = 10.6). No correlation was found between birth weight in the total study population and placental 11ß-HSD activity (Figure 5.5). This result along with the data for ALP and GNA correlations are summarised in Table 5.2. As for 11ß-HSD, no correlation was found with birth weight. No correlations were found between any enzyme and placental weight.

Table 5.1 Human 11ß-HSD *In-Vitro*: Study Details

Variable	N	Mean	Range	SEM
Length of gestation [weeks]	524	39.9	28.0 - 43.4	0.1
Birth Weight [kg]	524	3.38	1.24 - 5.02	0.01
Placental weight [g]	517	519	215 - 1048	5

Table 5.2 Human 11ß-HSD *In-Vitro*: Pearson's Correlation Coefficients

Enzyme	N	Birth Weight (p)	Placental Weight (p)
11ß-HSD	500	- 0.05 (ns)	- 0.02 (ns)
ALP	495	+ 0.01 (ns)	- 0.08 (ns)
GNA	497	+ 0.08 (ns)	+ 0.08 (ns)

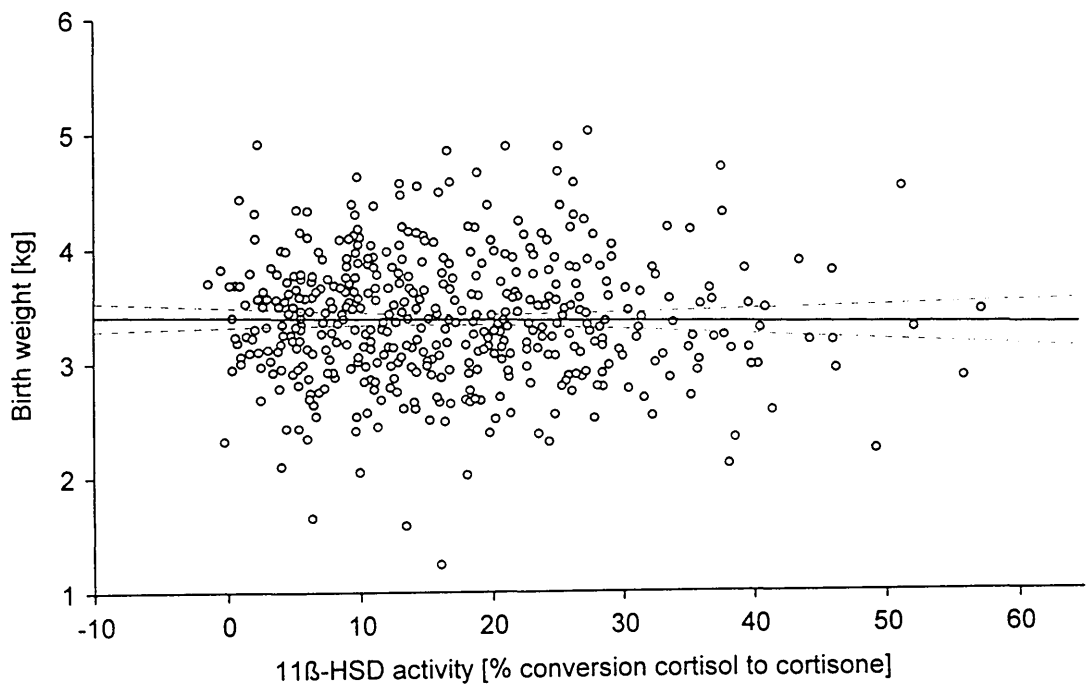


Figure 5.5 Human Birth Weight and 11ß-HSD Bioactivity *In-Vitro*

The figure plots net (duplicate mean minus blank) 11ß-HSD activity for 500 placentas prospectively collected in Southampton in collaboration with Professor Barker's group. The solid line through the data points represents the regression line while the dotted lines indicate 95% confidence intervals for the regression. Pearson's r = - 0.05, p is ns.

A few confounding factors are present. Firstly, 235 of the women had had a previous live birth. As birth weight tends to increase with successive pregnancies, this would serve to obscure any correlation of 11ß-HSD activity and birth weight. Also, 19 babies whose placental 11ß-HSD activity was available, had birth weights < 2500 g (19 had placental sampling), the traditional value separating frankly low birth weight babies from normals - this would again serve to obscure any correlation of 11ß-HSD and birth weight. There was however no difference in 11ß-HSD activity between the normal and low birth weight groups (data not shown). The low weight babies also had significantly lower gestational age than the normals (36.0 ± 0.7 weeks vs. 40.0 ± 0.1 weeks normals, $p < 0.0000001$). Nine of the 19 were small for gestational age, but showed no difference in 11ß-HSD activity when compared to those not small for gestational age. By categorising babies born within 38 - 42 weeks as either small for gestational age (birth weight $< 10^{th}$ percentile; $n = 41$) or normal ($n = 385$) and then looking at 11ß-HSD activity, no difference was found. Taking all of the above into account, i.e. analyse those with normal length of gestation (38 - 42 weeks), including only first deliveries between 10^{th} and 90^{th} percentiles (birth and placental weights), there was no correlation of 11ß-HSD activity and birth weight ($n = 150$).

As discussed in the Section on rat placental 11ß-HSD, a more relevant estimate of placental glucocorticoid metabolic capacity may be the total placental activity, obtained by multiplying the activity per gram placental homogenate with total wet placental weight. This showed a significant correlation with birth weight ($r = 0.16$, $p < 0.0005$, $n = 515$), but one has to bear in mind that placental weight was strongly positively correlated with birth weight (see above). This correlation is therefore meaningless.

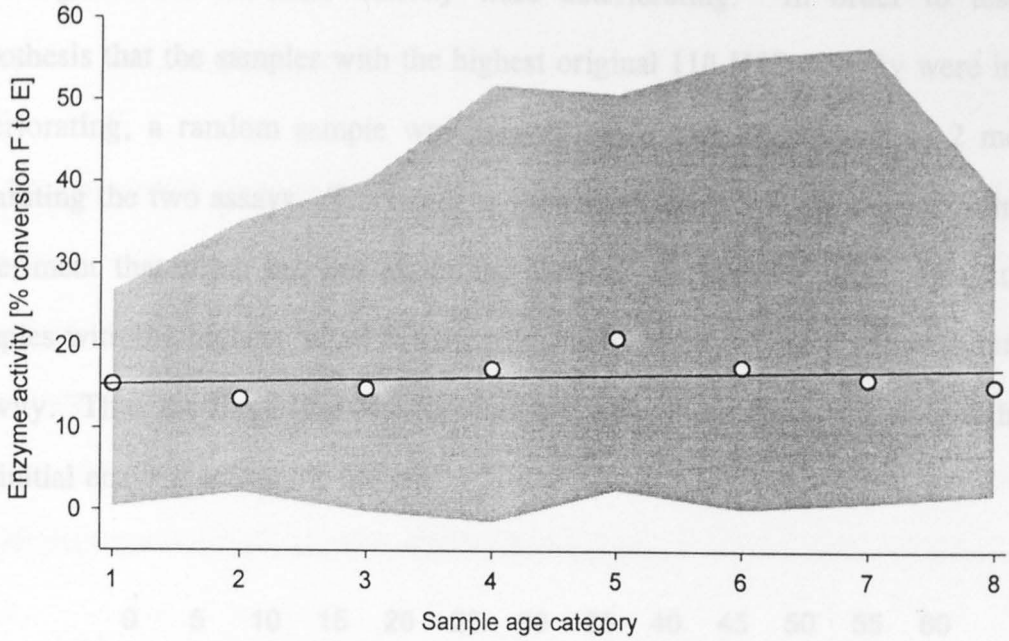


Figure 5.6 Long Term Storage of Human Placental 11ß-HSD

Samples have been categorised according to storage time (6 - 15 months) into 8 equally long time periods. The oldest category is 1 (to the left), the most recent samples in category 8. The points (fitted line) indicate mean 11ß-HSD activity for each category. The shaded area denotes the range (minimum - maximum) of 11ß-HSD activities in each category. Note the marked trend for less variability in the older sample categories when compared with the more recent categories. This is due entirely to lower maximum values, while the mean for each group is constant.

Finding no correlation of birth weight with term placental 11ß-HSD activity in humans was disappointing. Was there an explanation? Was it possible that the samples were not stable on long term storage? Was the pilot study on trophoblast stability flawed - too short term? Although there was no correlation between the age of the samples and 11ß-HSD activity (the youngest sample was 6 months old, and they spanned 15 months), analysing them in a slightly different way generated an interesting result. For this analysis, the samples were divided into 8 groups, all representing equally long time intervals. There was no difference in the mean 11ß-HSD activity in these groups, but there was a definite trend in the variability of the 11ß-HSD values (Figure 5.6). Thus the oldest samples had much less variability in 11ß-HSD activity. It therefore seemed that some of the samples, notably those

with high initial 11ß-HSD activity were deteriorating. In order to test the hypothesis that the samples with the highest original 11ß-HSD activity were in fact deteriorating, a random sample was assayed twice with approximately 2 months separating the two assays. The result is shown in Figure 5.7. It is clear from this experiment that some but not all of the samples are deteriorating. In fact, the samples with the highest initial activities (x - axis) seem to lose greatest amount of activity. Thus the fitted line departs more and more from the x = y dotted line as the initial enzyme activity is higher.

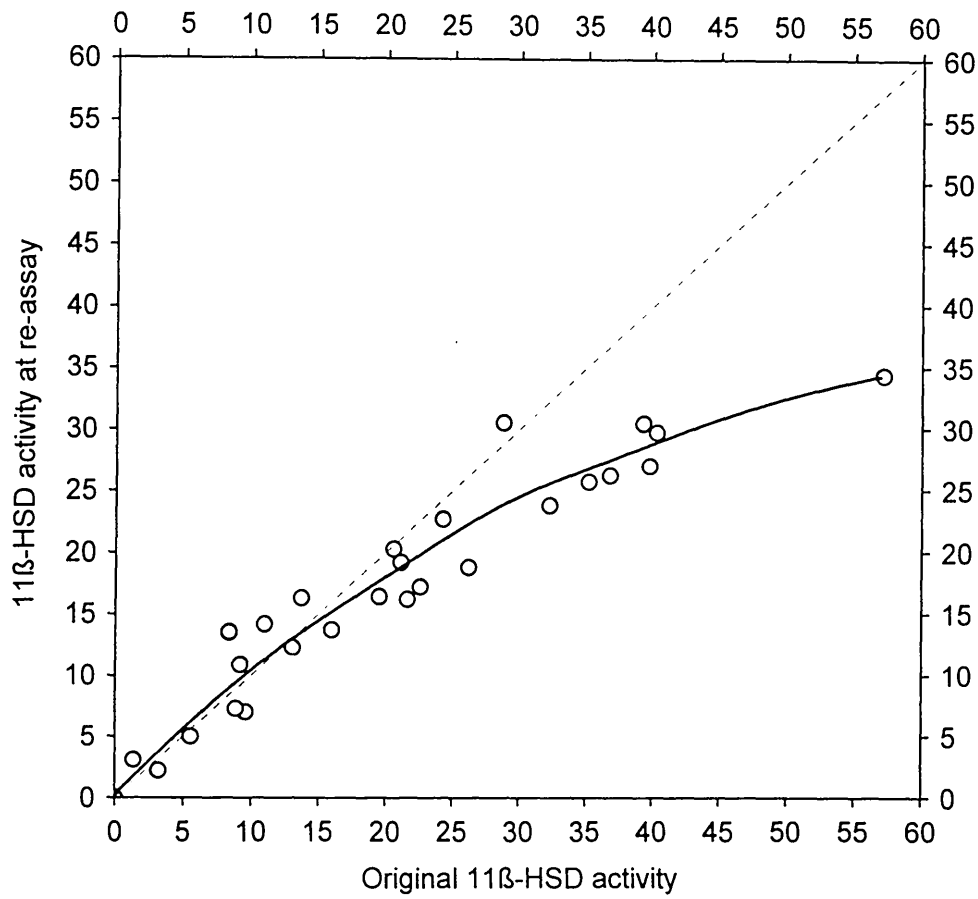


Figure 5.7 Repeat 11ß-HSD Assay of Stored Human Trophoblast

29 placentas were selected at random and assayed twice, approximately 2 months apart. The horizontal x - axis indicates 11ß-HSD activity at first assay and the vertical y - axis indicates 11ß-HSD activity at re-assay two months later. The solid line is "least squares" fitted through the data points while the dotted line indicates x = y.

5.4. DISCUSSION

The data presented here on cofactor preferences of human placental 11ß-HSD are in keeping with the notion that human placenta expresses predominantly an NAD dependent 11ß-HSD isoform (BROWN1993}. There seems to be considerable endogenous 11ß-HSD activity (without adding cofactor) which is in contrast to the observations on rat placenta *in-vitro* (Section 4.3.1). As in the rat placenta, some NADP dependent dehydrogenase activity was found.

No reductase activity was found in the current study confirming other researchers' results, namely that placental 11ß-HSD predominantly is an steroid inactivating dehydrogenase [Brown et al., 1993] protecting the human foetus from maternal glucocorticoids throughout pregnancy [Murphy et al., 1974; Pasqualini et al., 1970; Dancis et al., 1978; Beitins et al., 1973]. It is however not possible from the data presented here, to exclude the presence of 11ß-HSD-1, especially in light of positive immunohistochemistry and presence of mRNA for 11ß-HSD-1 in the rat placenta throughout gestation as shown in Section 4.3.3 (although in the rat as in humans, there is no detectable reductase bioactivity *in-vitro*).

Therefore, as the mRNA is translated into protein (positive immunohistochemistry in the rat) either the reductase component is inactive due to special intracellular conditions, or it acts as 11ß-HSD-2, as a dehydrogenase. The third possibility would be degeneration of the reductase with tissue homogenisation, since the reductase has previously been found to be more sensitive to tissue manipulation than the dehydrogenase, [Lakshmi et al., 1993; López Bernal et al., 1982a]. Finally it is possible that both isoforms are present and active, 11ß-HSD-1 as a

reductase and 11ß-HSD-2 a dehydrogenase, the latter being more active and thus using the current methodology, it would not be possible to detect any production of ^{3}H-cortisol as it is immediately converted back to ^{3}H-cortisone.

It should however be kept in mind that there seem to be important inter-species differences present, as in the baboon considerable reductase activity can be detected in the placenta at mid-gestation [Pepe & Albrecht 1984b], in contrast to data from humans *in-vitro*, *in-vivo*, and using *ex-vivo* perfusion [Brown et al., 1993; Blasco et al., 1986; Dancis et al., 1978; Murphy et al., 1974; Pasqualini et al., 1970; Beitins et al., 1973].

The lack of correlation of placental 11ß-HSD activity with birth weight was disappointing for several reasons. Firstly, the epidemiological data presented by Professor Barker's group on birth weight and ischaemic heart disease (including blood pressure) is very strong and has been repeatedly observed by that group in several different populations at different ages. The same observations have also been made by other authors in populations with distinct genetic background [Seidman et al., 1991; Gennser et al., 1988]. Secondly, although the mechanism is not proven, several lines of evidence point to a possible role of maternal glucocorticoids in this respect - see the rat placental 11ß-HSD data presented in Sections 4 and 1.1.3.

Further support comes from unpublished observations which show that the effect of 11ß-HSD inhibitors (reduction in birth weight), can be prevented by maternal adrenalectomy [Lindsay & Seckl 1994]. Finally, new data are emerging, indicating that 11ß-HSD might be a final common pathway by which more than one stimulus can induce offspring hypertension, since maternal protein malnutrition

during pregnancy, which reduces birth weight and results in hypertensive offspring [Langley & Jackson 1994], appears to impair placental 11ß-HSD activity [Phillips et al., 1994].

Of lesser concern was the fact that no correlation was found between placental weight and placental 11ß-HSD activity. The phenomenon of low birth weight and high placental weight has been inconsistent and sub-analysis of some of the studies has allowed identification of several patterns of birth and placental weights predicting hypertension (see Section 1 for further discussion). Thus, birth weight, although a crude measure of foetal growth is probably the prime index which should be used for the analysis.

Finding a highly significant, albeit weak ($r = 0.16$, $p < 0.0005$, $n = 515$) correlation of birth weight with "total placental" 11ß-HSD activity is not unexpected, but is meaningless, as "total placental" 11ß-HSD activity is obtained by multiplying 11ß-HSD activity per gram homogenate protein, with wet placental weight. As birth weight showed a strong and significant correlation with placental weight ($r = 0.60$, $p << 10^{-6}$), the correlation of "total placental" 11ß-HSD is attributable to the placental weight - birth weight correlation. Also, the protein content of the homogenate may not represent placental protein only (blood etc.) and areas not available for materno-foetal exchange, possibly devoid of 11ß-HSD, may have been included in the homogenate. Similarly, any correlations of placental weight with "total placental" 11ß-HSD activity will be meaningless.

However, as mentioned in the introduction to this Section, the problem encountered here (lack of correlation of birth weight and placental 11ß-HSD activity), is not entirely unexpected as there are important differences between the

methodology used in the rat experiments and those used in this Section on human placental 11ß-HSD *in-vitro*. The difference which is most likely to explain the discrepancy in correlations is the fact that placental 11ß-HSD is unstable on storage, and for the rat studies fresh placentas were used, while frozen-thawed trophoblast was used in this Section on human placenta.

Although part of the validation studies in this Section concerned storage of the placental tissue, they might not have been extensive enough. For example the storage experiment (Figure 5.3) looked at storage over 35 days while the placental trophoblast samples from Southampton were collected over a 15 month period, the youngest being 6 months old when assayed. Thus sample deterioration (after 35 days storage - which might then be accelerated) was possible and is in fact highly likely as judged by Figure 5.6, where the oldest samples seem to exhibit less variability of 11ß-HSD activity. This was then confirmed (Figure 5.7) by showing that the initially high 11ß-HSD activity samples, specifically deteriorated over a two month period, while the initially low activity samples remained unchanged.

Furthermore, although in the short term, the kinetics of the 11ß-HSD assay were similar using fresh and frozen specimens, that validation rests on a small number of sample placentas which had roughly similar (and low) activities. Thus even though assay conditions were on the linear part of the reaction progress curve, the variation might have been too small to enable differences to be detected over the course of a few days (bear in mind that the range of placental 11ß-HSD activities in the study itself was considerable, and age of specimens variable, ranging from 6 to 21 months).

Thus it is most likely that human placental 11ß-HSD is unstable on long term storage at -70 °C as other authors have previously suggested [Lakshmi et al., 1993; López Bernal et al., 1982a; Brown et al., 1993]. Possibly there are labile and stable parts of the enzyme, the loss of the labile form resulting in the initially high activity specimens losing more activity. Thus all the samples would "settle" at a similar low level of activity - as was indeed found.

This problem can only be resolved by using fresh human placental homogenates, which is impossible on the scale of this study. Therefore it is even more important to look at 11ß-HSD activity with the *ex-vivo* perfusion methodology where intact placentas are used. The results of such a study are described in Section 6. An important aspect of this later study is comparison of the activity of placental 11ß-HSD activity *ex-vivo* with that *in-vitro* in frozen-thawed stored specimens of the same placenta.

It is tempting to maintain that the lack of correlation of birth weight with either ALP or GNA signifies that the correlation of birth weight with 11ß-HSD activity described for the rat is specific and not a general phenomenon. In the light of the results on human 11ß-HSD described/discussed here, that is however not appropriate.

6. HUMAN PLACENTAL 11ß-HSD EX-VIVO

6.1. INTRODUCTION

Several recent epidemiological studies have consistently implicated prenatal events in the development of common adult cardiovascular and metabolic disorders. Thus, low birth weight is strongly predictive of the later occurrence of hypertension [Barker et al., 1990; Law et al., 1993]. The link between low birth weight and hypertension has been demonstrated in several distinct populations and holds in children [Law et al., 1991; Cater & Gill 1984; Barker et al., 1989a], adolescents [Seidman et al., 1991] and adults [Gennser et al., 1988; Barker et al., 1989a; Barker et al., 1990]. Indeed, the well-recognised "tracking" of blood pressure from infancy to adulthood in man [Lever & Harrap 1992] clearly implicates early events in the determination of blood pressure throughout life.

The mechanisms linking prenatal events and later disease are unknown, although maternal malnutrition has been advocated [Barker et al., 1993a]. We advanced the hypothesis that glucocorticoid excess *in-utero* might be important [Edwards et al., 1993a], as glucocorticoids retard foetal growth and treatment of pregnant rats with dexamethasone resulted in elevated offspring blood pressure [Benediktsson et al., 1993]. The mode of glucocorticoid action is unclear, but steroids, including glucocorticoids, exert well-recognised pre- or perinatal effects determining tissue maturation [Slotkin et al., 1992b; Slotkin et al., 1992a] and permanently programme anatomy, biochemistry and function [Seckl 1994].

The foetus is thought to be protected from maternal glucocortcoids by the placental enzyme 11ß-hydroxysteroid dehydrogenase (11ß-HSD) [Murphy et al., 1974; Beitins et al., 1973]. In support of our hypothesis we found that placental 11ß-

HSD shows considerable natural variation in the rat, with the lowest activity (and presumably the greatest foetal exposure to maternal glucocorticoids) in the smallest offspring (Section 4) [Benediktsson et al., 1993]. Moreover, when pregnant rats are treated with an 11ß-HSD inhibitor, birth weight is reduced and the adult offspring blood pressure raised [Lindsay et al., 1994b].

When the same *in-vitro* methodology that had been used in the rat experiments was applied to human placentas (Section 5), the results were very disappointing in that no correlation was found between birth weight and placental 11ß-HSD activity in frozen-thawed term trophoblast samples. The explanation for this was most likely (as discussed in Section 5.4) to be instability of 11ß-HSD when stored at -70 °C. It was therefore of key importance to look at 11ß-HSD function in fresh placentas, preferably using methodology where the question of which 11ß-HSD isoform to look at, could be bypassed, and importantly, where placental anatomy (the physiological glucocorticoid barrier) was intact.

For this purpose, *ex-vivo* dual circuit cotyledon perfusion of freshly isolated intact human placentas was undertaken. This methodology has been extensively used for looking at various aspects of placental physiology including glucocorticoid metabolism [Addison et al., 1993; Addison et al., 1991; Dancis et al., 1980; Dancis et al., 1978; Dodds et al., 1993; Levitz et al., 1978; Smith et al., 1988]. The studies on glucocorticoids have used both open- and re-circulating maternal and foetal perfusates and have looked at both naturally occurring and synthetic glucocorticoids. All studies agree on extensive inactivation of the relevant steroids, all however used supraphysiological concentrations (μM range) of free steroid (the concentration used was not clearly stated in some), and some have in fact invoked other enzymes than 11ß-HSD in the metabolism of cortisol [Dodds et al., 1993] and prednisolone [Addison et al., 1991] in human placentas, casting doubt on the crucial barrier role of 11ß-HSD.

Before carrying out a formal study on human placental 11ß-HSD activity and birth weight using this methodology, it was necessary to carry out validation studies, for example looking at the behaviour of cortisol transport, the viability of the preparation, and to address the question whether 11ß-HSD was in fact the placental glucocorticoid barrier.

6.2. METHODS

6.2.1. Subject Selection

To develop and validate the *ex-vivo* perfusion methodology, term placentas were taken at random. Pregnancies complicated by hypertension, smoking, hepatitis B or HIV infections were excluded.

To study the relationship between birth weight and placental 11ß-HSD activity, 20 consecutive singleton, term, first pregnancies were enrolled during the 3rd trimester. All women gave informed consent to the study, which was fully approved by the local Ethics of Medical Research Committee. Exclusion criteria were smoking, alcohol consumption, drug abuse, chronic illness including bronchial asthma, the presence of high risk infections (hepatitis B, HIV), regular medication of any kind or pregnancy-induced hypertension (blood pressure > 140/90 mmHg or increase of > 30/15 mmHg compared to the first trimester). Data are presented for 16 subjects, whose placentas were successfully perfused. Two were excluded because the placentas were very torn at delivery and had gross fluid leaks, and two had high foetal circuit pressures (see below). Birth weight was recorded to the nearest 5 g in a standardised manner within 1 h of delivery and total wet weight of the placenta was measured to the nearest gram after careful removal of the umbilical cord and membranes.

6.2.2. Perfusion System

The methodology has been described in detail in Section 2.8. Briefly it was based on the description by Schneider [Schneider et al., 1972; Schneider & Huch 1985]. On delivery the placenta was immediately immersed in ice-water (protected by a plastic bag). Handling of the placenta at delivery was performed with extreme care and minimised. A suitable pair of chorionic vessels supplying a well-defined and macroscopically-intact cotyledon were cannulated and sutured in place. Occasionally, when vessel anatomy dictated, collaterals to adjacent cotyledons were tied off. The foetal circulation was started slowly, increasing to 6 ml/min (higher foetal flow rates resulted in foetal fluid loss and altered morphology on electron microscopy - this flow rate compares with several other investigators). This allowed the perfused cotyledon to be identified, isolated and embedded in a custom-made plexiglas perfusion chamber.

The congruent area on the maternal side of the cotyledon, identified by virtue of slight blanching, was pierced to a depth of 1 - 2 cm using two 21G cannulae and the maternal circulation separately perfused at 10 ml/min. Both perfusates were modified Kreb's buffer (the foetal perfusate also contained dextran), the maternal gassed with 95% O_2:5% CO_2, the foetal with 95% N_2:5% CO_2 both warmed to 37 °C. This gas composition used for the foetal perfusate is believed to be needed in order to retain morphological and biochemical integrity of the preparation [Kuhn et al., 1988]. The two separate circulations were driven by a multi-channel peristaltic pump. To maintain temperature, the entire perfusion chamber was housed in an incubator at 37 °C (see diagram of setup: Figure 2.3). No albumin or corticosteroid binding globulin was included in the perfusates as cortisol metabolism by 11ß-HSD is not affected by either protein [Dancis et al., 1978].

Circulatory overlap was assessed by visualisation of blanching on the maternal side after starting the foetal circuit and by observing the step-up of pO_2 across the foetal cotyledon using an blood gas analyser. The pressures in both circuits were

continuously recorded by in-line (afferent) transducers and computer-assisted monitoring. The temperature of both maternal and foetal perfusates was monitored using in-line thermocouples and a digital thermometer. In addition to timed collections of the foetal output, flow meters were included in the foetal afferent and efferent lines to allow immediate detection of any fluid loss from the foetal circuit.

Perfusion was considered unsuccessful if no O_2 step-up across the cotyledon (indicating no materno-foetal circuit overlap) was observed, if foetal circuit pressure exceeded 60 mmHg, (this has been suggested as essential for preservation of placental ultrastructure [Jauniaux et al., 1991]) or if a foetal circuit fluid leak was observed. The wet weight of the perfused cotyledon was obtained at the end of the experiment, after the perfused area had been defined by injecting ink into the foetal circulation.

6.2.3. Sampling and Analytical Methods

This has been described in detail in Section 2.8. Briefly, foetal outflow samples were collected over either 3 or 5 min intervals, and in the case of the prospective study over two consecutive 5 min intervals at steady state, and data averaged for analysis. ^{14}C-antipyrine and steroids were extracted using a modification of the method described by Cannell [Cannell et al., 1982] for extracting steroids from plasma. Thus the foetal effluent samples, were centrifuged to pellet debris, acidified to pH 2 (checked with pH paper for each sample) with HCl, extracted through Sep-pak Plus cartridges (Waters, UK) and eluted with ethyl acetate. Cortisol, cortisone and antipyrine were separated and quantified using isocratic HPLC as described on page 85 and in Figure 2.1. Some of the perfusions did not include any labelled cortisol, but only cold; these were also analysed with HPLC but including standards with known amounts of steroids in each run allowing concentration of cortisol in the samples to be calculated as described in detail in Section 2.8.3).

The activity of 11ß-HSD (the efficiency of cortisol inactivation) was determined in the conventional way as percent conversion cortisol to cortisone ($[^{E}/_{(E+F)}]$ x 100), in the foetal effluent per unit perfused cotyledon weight and also for total placental weight. Clearances were calculated as: (foetal concentration x perfusion flow rate)/maternal concentration. The glucocorticoid clearance index was total corticosteroid clearance/antipyrine clearance.

6.3. RESULTS

6.3.1. Validation and Viability of the Preparation

The placenta suffers an ischaemic insult at delivery and it has become customary to allow 30 - 60 min recovery period (no precise agreed guidelines exist in the literature as to which specific time to pick) after the perfusion has started, in order to allow various metabolic functions to recover. For the series of experiments presented here it was, in relation to this, felt appropriate to examine how much time was needed to allow near complete washout of endogenous glucocorticoids retained in the placenta (cortisol and cortisone). The pattern of washout of cortisol and cortisone is shown in Figure 6.1, which shows that both steroids are undetectable after 40 minutes.

Further support for choosing at least 40 min as the washout/recovery period for all further perfusions came from studying the profile of lactate production by the perfused cotyledon in this setup (Figure 6.2). This study (n = 5) showed that lactate reached a nadir before 40 min and did not rise again during the perfusion period.

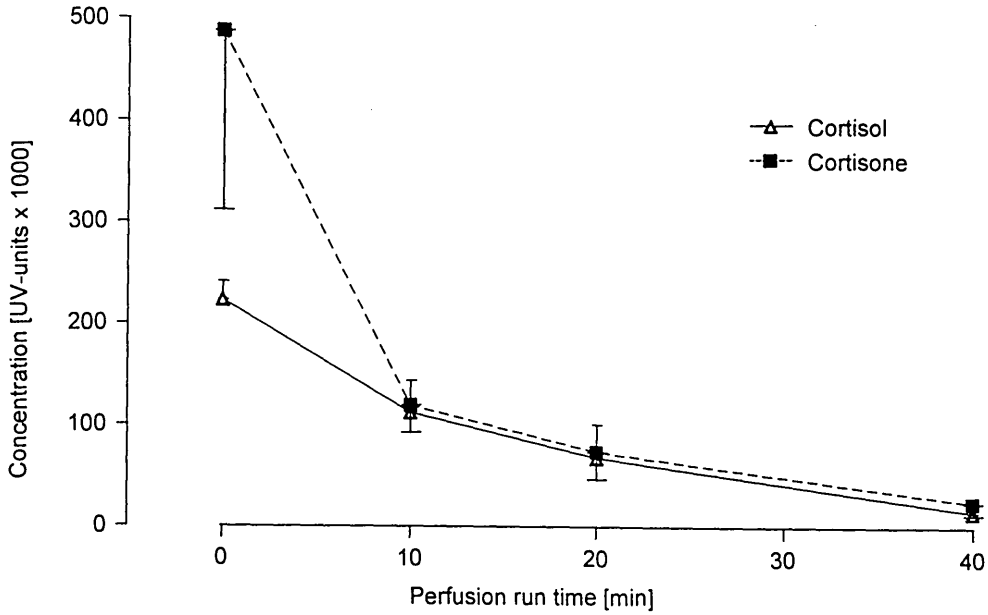


Figure 6.1 Washout of Endogenous Glucocorticoids

Cortisone concentration at t = 0 (which is ca 15 minutes following delivery, see later) are higher than cortisol and by 40 minutes most endogenous cortisol and cortisone has been washed out. Points represent means and bars ± SEM (n = 3-4)

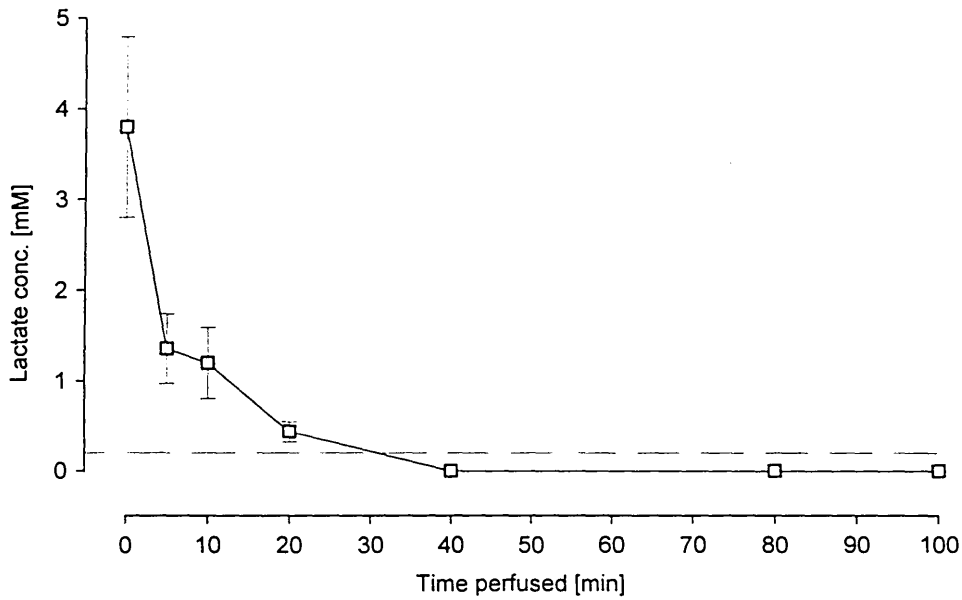


Figure 6.2 Pattern of Cotyledon Lactate Washout/Production

The figure shows lactate concentration (mM) in foetal effluent with progress of perfusion. Lactate levels become undetectable before 40 min and do not rise during the following period of study (up to 100 min). The horizontal dashed line identifies detection limit of the assay. Each point represents mean of data from 5 perfusions while bars represent ± SEM.

To confirm that the preparation was metabolically viable with respect to 11ß-HSD activity (or efficiency) for an extended period of time following delivery, 3 placentas were perfused with 50 nM cold cortisol only, on the maternal side. The foetal effluent was collected over 3 min at intervals throughout the perfusion, including 2 collections during the washout period (t < 40 min, i.e. estimating 11ß-HSD activity *in-vivo* from the ratios of endogenous cortisol and cortisone). As can be seen from Figure 6.3, 11ß-HSD activity remains constant throughout the 3 h period with no differences between individual samples detected by ANOVA. Apart from confirming the viability of the preparation, this has further implications, namely that the *ex-vivo* estimated 11ß-HSD efficiency is representative of *in-vivo* behaviour of 11ß-HSD. As only cold cortisol was used, not all the samples could be analysed due to background interference on the HPLC trace, thus each point n = 2 - 3, although 3 placentas were perfused.

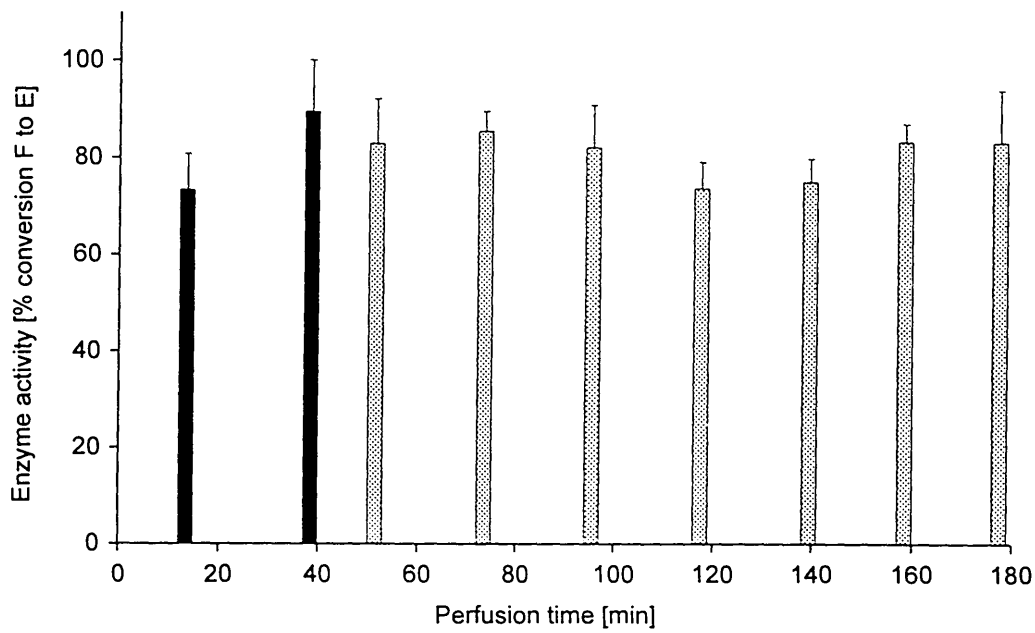


Figure 6.3 Stability of 11ß-HSD Activity in the Perfused Human Cotyledon

The activity of 11ß-HSD *in-vivo* (estimated from the endogenous levels of glucocorticoids, the first two left columns - solid) is no different from the activity *ex-vivo* (ANOVA), i.e. after starting perfusion with 50 nM cortisol on the maternal side (beyond 40 min - dotted columns). Placental 11ß-HSD in the perfused setting remains viable for 180 min. The width of the bars equals the collection period and represents the mean for 2 - 3 perfusions while bars represent mean + SEM.

Further data on the viability of the preparation came from histological specimens, taken immediately after perfusion (3 h) from the perfused cotyledon and adjacent non-perfused tissue. These were processed for light and electron microscopy, which confirmed ultrastructural integrity of perfused trophoblast cells, microvilli and subcellular organelles. Sections post 3 h perfusion were identical to sections from fresh placentas. Unperfused parts of the same placentas showed classical degenerative changes [Kaufmann 1985] with swelling of cells, vacuolation, swollen mitochondria and endoplasmic reticulum (Figure 6.4 and Figure 6.5).

The final validation experiments addressed the kinetics of cortisol to cortisone conversion and transplacental passage. For this purpose 50 nM cold cortisol along with a tracer of ^{3}H-cortisol (< 0.5% of total cortisol) was used since using cold steroid alone hampered analysis of samples due to background "noise" or interference by unknown substances in the perfusates. However, there was the possibility of an "isotope" effect, the placenta handling ^{3}H-cortisol differently from cold cortisol. To examine this, a 40 min washout period was allowed as before, followed by perfusion with exogenous cortisol mixture from 40 to 80 min. The foetal effluent was collected over continuous 5 min periods and the results expressed as concentration of tracer (^{3}H-cortisol and ^{3}H-cortisone) in the perfusates in counts per ml (Figure 6.6).

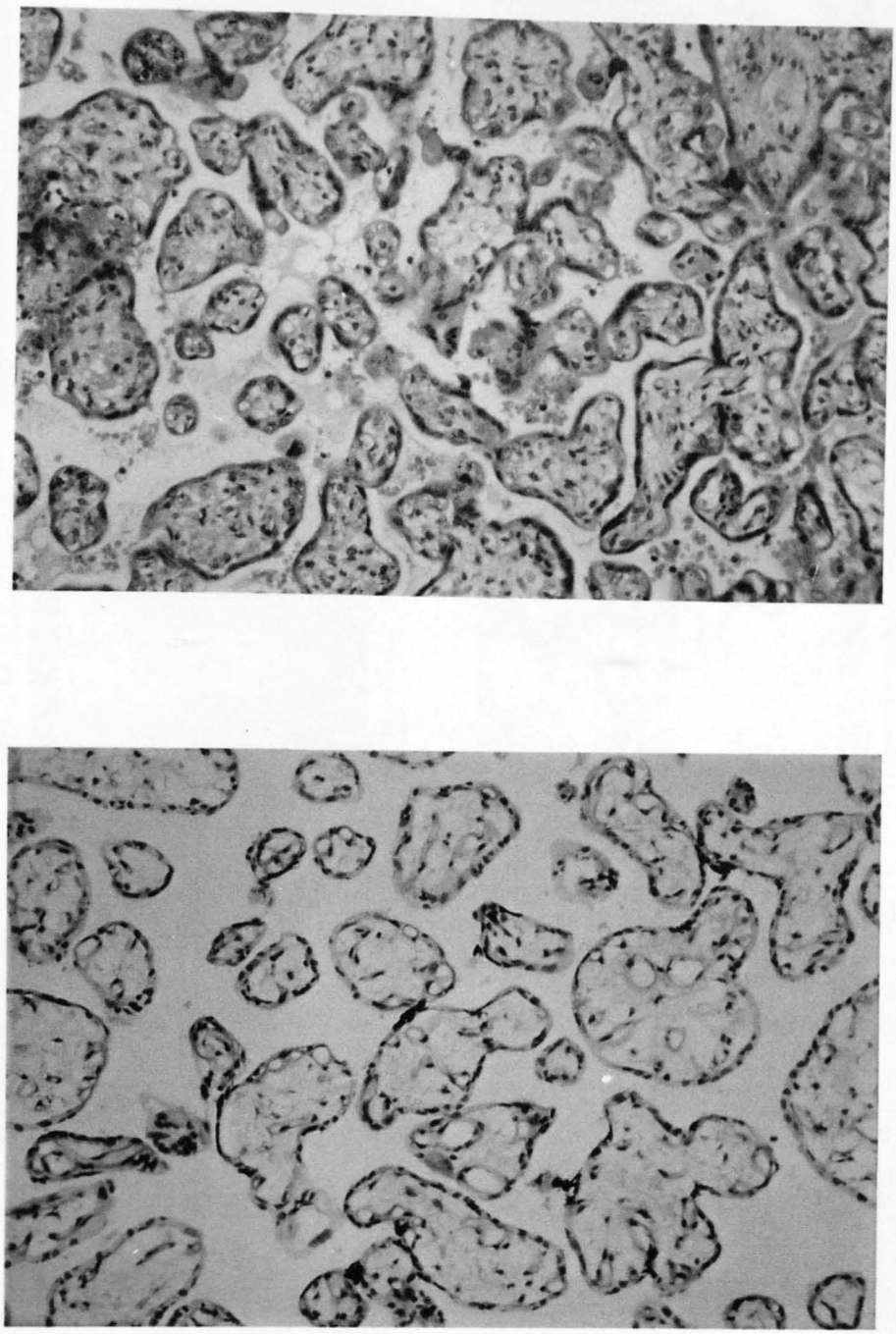


Figure 6.4 Light Microscopy Photomicrographs of Human Placenta

The figures show a remarkable conservation of morphology after 3 h perfusion (bottom) as compared to unperfused section of the same placenta (top).

Figure 6.5 Electron Microscopy Photomicrographs of Human Placenta

Part A shows trophoblast from an unperfused fresh placenta fixed within 20 min of delivery. Part B shows a section of trophoblast from a placenta which has been perfused as detailed in the text for 3 h. Note absent vacuolation and normal looking endoplasmic reticulum and mitochondria, while in Part C which is a section from an adjacent non-perfused cotyledon of the same placenta vacuolation, and swollen subcellular organelles can be seen.

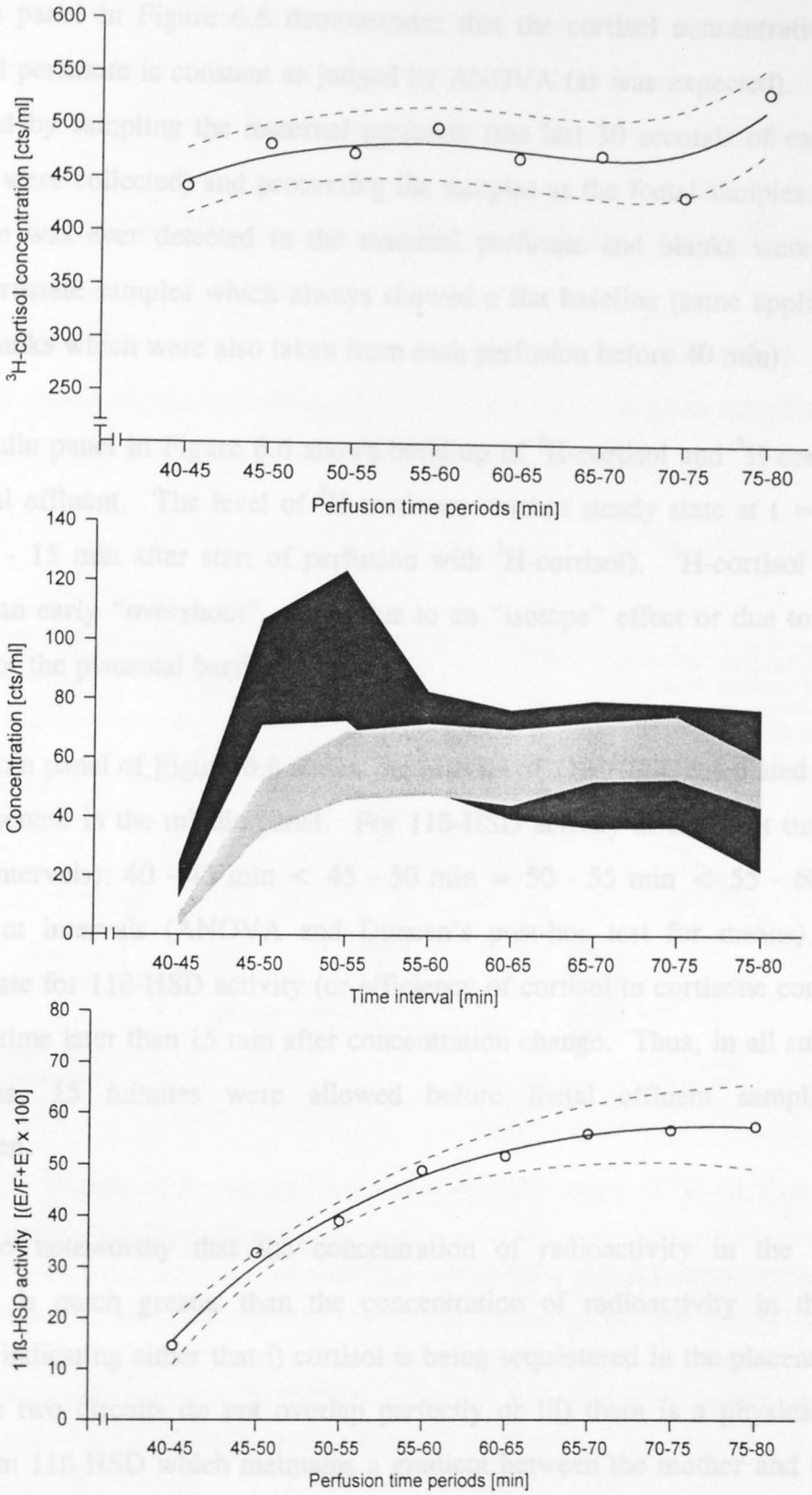


Figure 6.6 Kinetics of ^{3}H-Cortisol Transfer and Metabolism During Perfusion

Top is maternal circuit concentration of ^{3}H-cortisol. Middle is foetal effluent counts/ml of cortisol (black area outlines mean ± SEM) and cortisone (lightly shaded area). Bottom is 11ß-HSD activity. Solid lines are fitted through the mean (circles, n = 4) while the dotted lines are fitted through ± SEM.

The top panel in Figure 6.6 demonstrates that the cortisol concentration in the maternal perfusate is constant as judged by ANOVA (as was expected). This was estimated by sampling the maternal perfusate (the last 30 seconds of each 5 min interval were collected) and processing the samples as the foetal samples. No ^{3}H-cortisone was ever detected in the maternal perfusate and blanks were "pre 40 min" perfusate samples which always showed a flat baseline (same applied to the foetal blanks which were also taken from each perfusion before 40 min).

The middle panel in Figure 6.6 shows build-up of ^{3}H-cortisol and ^{3}H-cortisone in the foetal effluent. The level of ^{3}H-cortisone reaches steady state at t = 50 - 55 min (10 - 15 min after start of perfusion with ^{3}H-cortisol). ^{3}H-cortisol however showed an early "overshoot", either due to an "isotope" effect or due to physical leakage of the placental barrier.

The bottom panel of Figure 6.6 shows the activity of 11ß-HSD, calculated from the data presented in the middle panel. For 11ß-HSD activity at different time points (5 min intervals): 40 - 45 min < 45 - 50 min = 50 - 55 min < 55 - 60 min = subsequent intervals (ANOVA and Duncan's post-hoc test for means). Thus, steady state for 11ß-HSD activity (or efficiency of cortisol to cortisone conversion) was any time later than 15 min after concentration change. Thus, in all subsequent perfusions, 15 minutes were allowed before foetal effluent sampling was undertaken.

It is also noteworthy that the concentration of radioactivity in the maternal perfusate is much greater than the concentration of radioactivity in the foetal effluent, indicating either that i) cortisol is being sequestered in the placental tissue or ii) the two circuits do not overlap perfectly or iii) there is a physical barrier apart from 11ß-HSD which maintains a gradient between the mother and foetus in spite of the lipid soluble nature of steroids (see later).

Details of sample preparation and recovery of radioactivity in the foetal perfusate samples are depicted in Figure 6.7. Note that less than 0.3% of total radioactivity

is lost in the water soluble waste suggesting that no enzymes (sulphatases etc.) are metabolising cortisol to form water soluble/conjugated steroids to any significant extent in this preparation. The samples used for these analyses were foetal effluent perfusates, the maternal circulation having been perfused with 50 nM ^{3}H-cortisol. For the rest of the recovery experiments, foetal effluent perfusates were collected, pooled and subsequently divided into 15 ml samples, spiked with ^{3}H-cortisol and cold cortisol to approximately 10^{6} cpm and approximately 200 nM respectively. The total counts were obtained by drying down samples in glass scintillation vials and re-dissolving in scintillant before counting in a ß-counter.

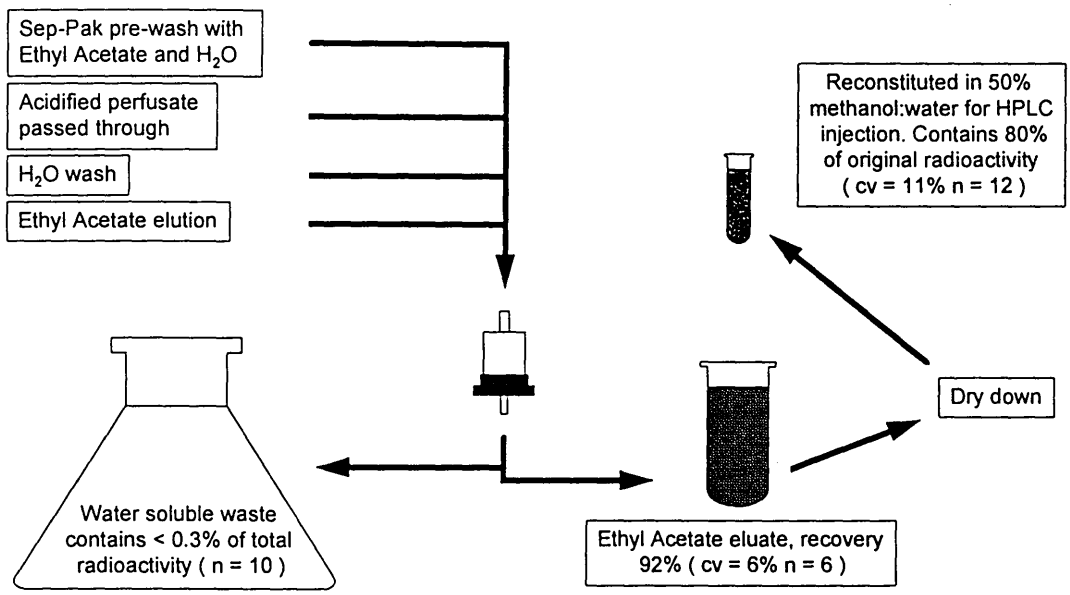


Figure 6.7 Details of Perfusate Extraction and Recovery of ^{3}H-cortisol

The figure shows details of sample preparation and the extraction recovery for cortisol (Section 2.8.3). The samples were foetal perfusates, the maternal circulation being perfused with 50 nM cortisol ("high normal" free physiological concentration). The passage through the Sep-pak Plus was driven by a vacuum pump, enabling 24 samples to be processed simultaneously. Samples were dried down under a gentle stream of air at 37°C. Using air as opposed to nitrogen for drying down did not affect the ratio of cortisol and cortisone, the only steroids in the samples

6.3.2. The Barrier Role of Human Placental 11ß-HSD

Does placental 11ß-HSD control the access of maternal glucocorticoid to the human foetus *in-utero*? This has been suggested by several authors [Murphy et al., 1974; Dancis et al., 1978; Beitins et al., 1973; Dormer & France 1973], but recently, other enzymes (20α/ß-hydroxysteroid dehydrogenase, 5ß-reductase) have been proposed as contributors to placental metabolism of glucocorticoids [Addison et al., 1991; Dodds et al., 1993]. These latter studies used *ex-vivo* placental perfusion with recirculating perfusates and pharmacological concentrations of cortisol and the synthetic glucocorticoid prednisolone. The physiological relevance of these studies is therefore unclear. First, the use of pharmacological concentrations may have resulted in metabolism by enzymes normally quiescent. Secondly, synthetic glucocorticoids may be protected from metabolism by specific structural alterations (an example is dexamethasone which is metabolised by NAD-preferring 11ß-HSD but not NADP-preferring 11ß-HSD [Siebe et al., 1993]). Finally, the practice of recirculating perfusates will subject the primary product of active glucocorticoid metabolism to further metabolism.

The data presented in the last Section, regarding recovery of radioactivity, add to confidence with regard to the proposal that 11ß-HSD is of primary importance in inactivating cortisol, as 92% of the radioactivity was recovered from the extraction procedure and no radioactivity was lost in the water soluble waste. Thus we could be confident that while perfusing the preparation with a "physiological free concentration of cortisol" [Meulenberg & Hofman 1990], all potential metabolites (see Figure 1.5) would be present in the sample injected onto the HPLC column. But was the isocratic HPLC employed able to separate the various potential metabolites?

To look at that, samples (cold) of all the potential metabolites were purchased (Sigma UK) and run against cortisol and cortisone on HPLC, using as always flow rate of 1.3 ml/min and 50% methanol:water as mobile phase. The result is depicted in Figure 6.8, which shows clearly that none of the potential metabolites co-elutes with either cortisol or cortisone. 20α- and 20ß-dihydrocortisol might have produced a shoulder on the cortisone and cortisol peaks respectively. In fact there were never any convincing shoulders on the HPLC traces. A typical HPLC trace of ^{3}H-cortisol and ^{3}H-cortisone is shown in Figure 6.9, which clearly shows that cortisone is the only metabolite of cortisol in this setting. This was true for a range of cortisol concentrations, from low (20 nM) to high physiological (200 nM) and also during inhibition of 11ß-HSD with carbenoxolone or glycyrrhetinic acid.

It had been noted (see previous Section) that a materno-foetal concentration gradient of cortisol was apparent across the placenta. In order to look at this further, 4 placentas were perfused, first for 40 min without steroid, then from 40 - 70 min with 20 nM cortisol, from 70 - 100 min with 50 nM cortisol and then from 100 - 130 min with 200 nM cortisol (i.e. low normal physiological free concentration - high normal physiological free concentration and finally very high physiological or pharmacological concentration). The perfusions also included stepwise increasing concentrations of ^{14}C-antipyrine, which is a small inert lipid soluble salt which doesn't interfere with cortisol metabolism [Dancis et al., 1978]. The purpose of including antipyrine was to provide a reference substance which, as cortisol, was lipid soluble, allowing calculation of steroid clearance relative to antipyrine for comparison with the literature. Two samples were taken at steady state (for both antipyrine and steroids), which were the last two 5 minute periods before the concentration was changed. The data from both 5 min periods were combined for analysis.

Perfusion of the maternal circuit with 20 nM, 50 nM and 200 nM cortisol produced a linear increase in the transfer of total glucocorticoid across the placenta at steady state (cortisol + cortisone in the foetal circulation) and also in antipyrine transfer as can be seen in Figure 6.10, which shows concentration of radioactivity in both the maternal and foetal circuits at each time point. Note that the fitted line for the antipyrine data intercepts both axes very close to zero whereas that is not the case for cortisol. This probably means that there is initial sequestration of steroids, at maternal concentrations below 20 nM, such that no steroid transfer is observed.

Again, as in Figure 6.6, there was a concentration gradient for both substances across the placenta, and the magnitude of this gradient (i.e. the total corticosteroid clearance = foetal concentration/maternal concentration x foetal flow rate) did change as the concentration of cortisol in the maternal circuit was altered. Thus, at 20 nM maternal cortisol, the total steroid clearance is 0.27 $\pm$ 0.08; at 50 nM it is 0.45 $\pm$ 0.13 and at 200 nM it is 0.61 $\pm$ 0.09. The antipyrine clearance does however not change to the same extent, resulting in an increase in the relative corticosteroid clearance (Figure 6.11), probably reflecting slight differences in lipid solubility of steroid and antipyrine. Figure 6.11 also shows that although the absolute and relative corticosteroid clearances increased with increasing maternal concentration, the efficiency of 11ß-HSD remained constant over this wide range of concentrations, indicating considerable functional reserve.

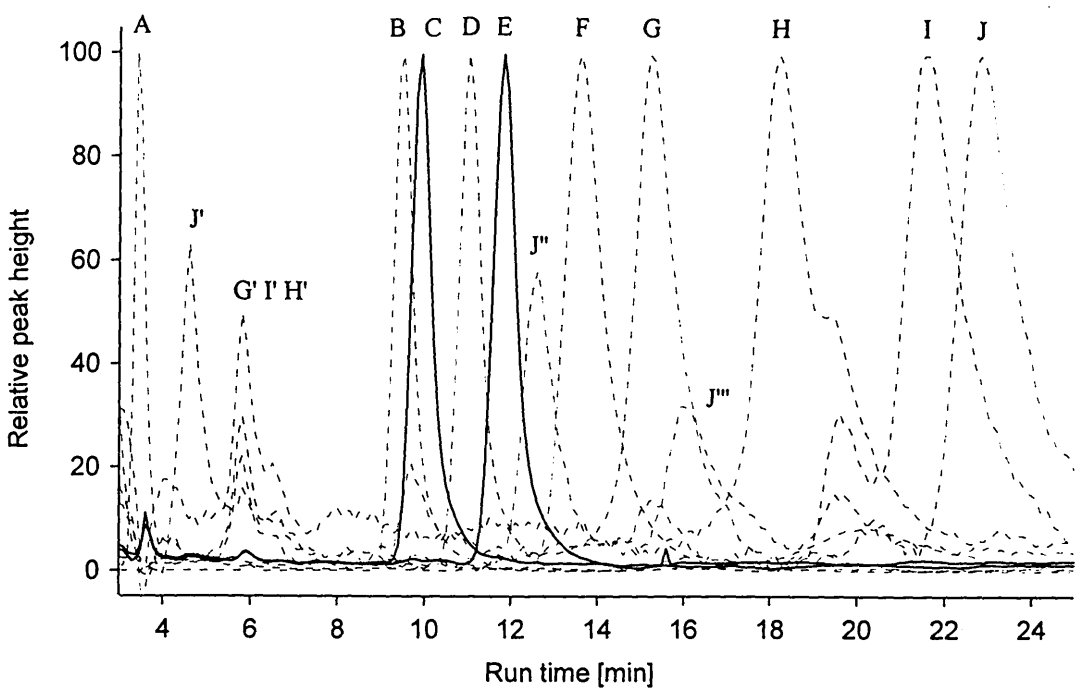


Figure 6.8 Elution Profiles of Cold Potential Cortisol Metabolites on HPLC

The flow rate was 1.3 ml/min and mobile phase was 50% methanol/water, column was reverse phase µ-Bondapak-C18. Note good separation of all peaks. Some substances (for example J) showed multiple minor peaks (identified by '), probably due to impurities/degeneration. Cortisol (E) and cortisone (C) shown in solid, other in dotted lines. A: 6ß-hydroxycortisol; B: 20α-dihydrocortisol; D: 20ß-dihydrocortisol; F: 11-hydroxyandrostenedione; G: 5α-dihydrocortisol; H: 5ß-dihydrocortisol; I: tetrahydrocortisol; J: allo-tetrahydrocortisol.

In separate studies, the maternal circuit was perfused with 50 nM cortisol with and without the 11ß-HSD inhibitor glycyrrhetinic acid at three concentrations. The washout period was 0 - 40 min as before, followed by perfusion with cortisol only (40 - 70 min), then co-perfusion of cortisol and glycyrrhetinic acid 10^{-8} M, (70 - 100 min) then 10^{-6} M (100 - 130 min) and finally 10^{-4} M (130 - 160 min). Samples were taken over two 5 min periods at steady state (+ 20 min), analysed separately and data combined for analysis.

Co-perfusion of cortisol with glycyrrhetinic acid resulted in marked and rapid inhibition of cortisone production by the placenta, so that most of the cortisol perfused on the maternal side was transferred intact to the foetal circulation (Figure

6.12). IC_{50} was approximately 3 x 10^{-6} M which is similar to that previously reported for intact renal tubular cells which also express 11ß-HSD-2[Monder et al., 1989]. The other commonly used inhibitor of 11ß-HSD is carbenoxolone, a water soluble synthetic hemi-succinate derivative of glycyrrhetinic acid. For the sake of completion, one perfusion was undertaken with 50 nM cortisol and carbenoxolone co-perfused at 10^{-4} M. The result is shown in Figure 6.13, which shows that while approximately 85% (in this placenta) of cortisol is metabolised, addition of carbenoxolone to the maternal perfusate permits all the cortisol to pass unmetabolised through the placenta from mother to foetus.

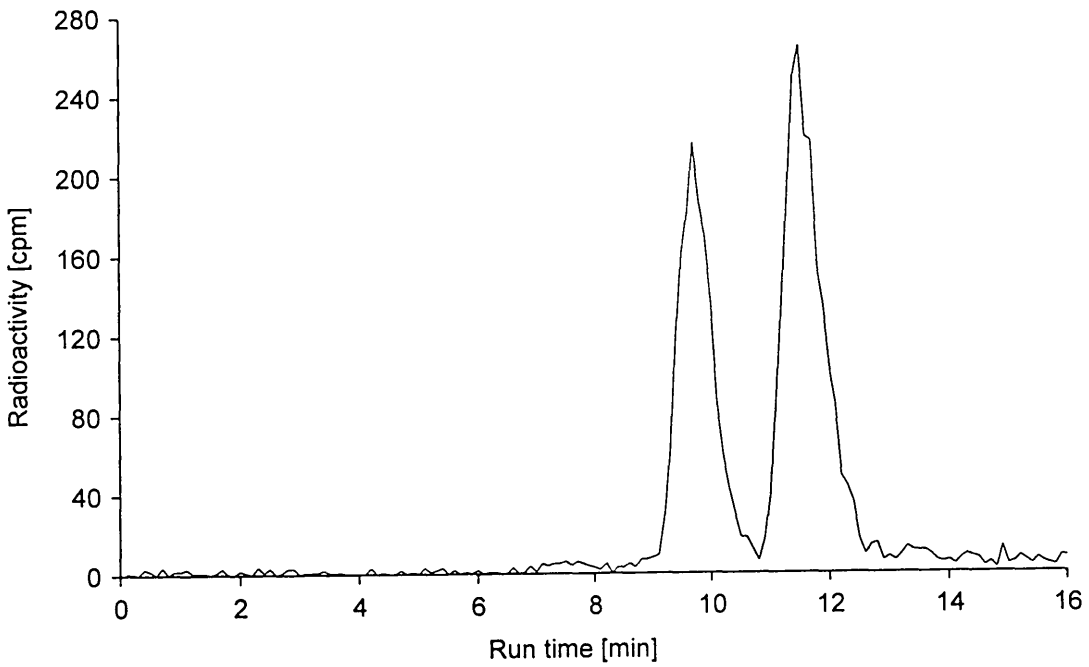


Figure 6.9 Typical HPLC Trace of a Foetal Effluent Perfusate Sample

The peak to the left is ^{3}H-cortisone and the one to the right is ^{3}H-cortisol, both elution times checked against authentic cold standards. No peaks were found beyond 16 min.

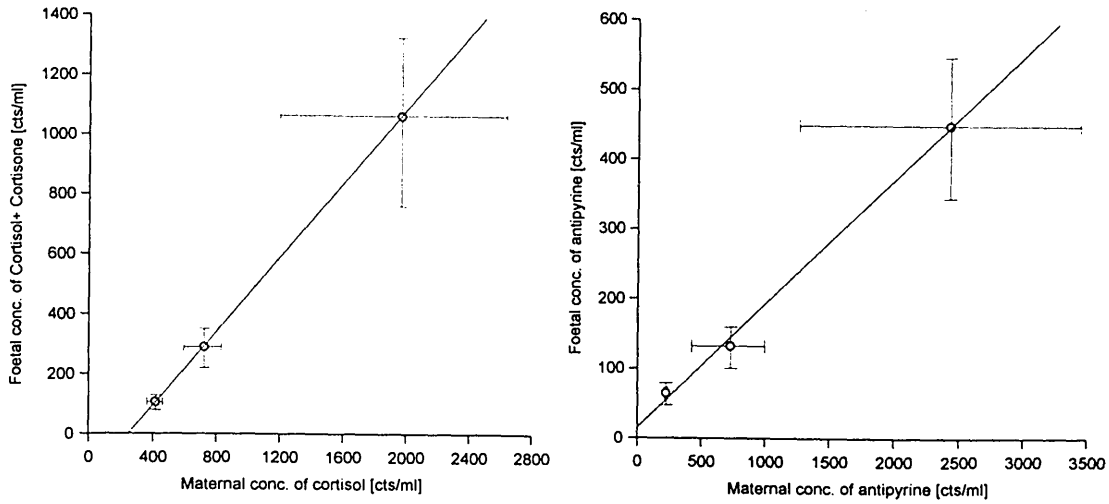


Figure 6.10 Maternal Concentration and Transfer of Cortisol and Antipyrine

To the left are data on ^{3}H-cortisol transfer, the horizontal axis showing maternal cts/ml and the vertical axis cts/ml recovered in the foetal effluent. Bars are mean ± SEM for 4 perfusions. The corresponding ^{14}C-antipyrine data are on the right.

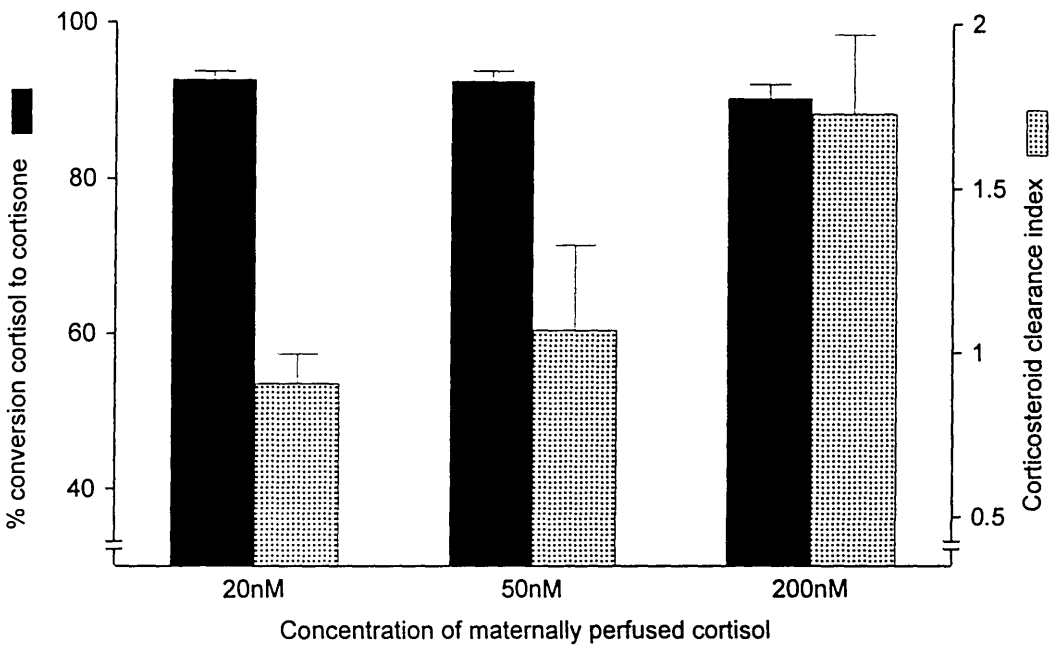


Figure 6.11 Corticosteroid Clearance Index and 11ß-HSD Activity

The black columns show a constant % conversion cortisol to cortisone over a wide range of maternal cortisol concentrations (left vertical axis) while the lighter shaded bars show the increase in relative corticosteroid (cortisol + cortisone) clearance (right vertical axis) over the same range of maternal steroid concentrations. Columns are means for 4 perfusions while bars indicate + SEM. Clearance index is steroid clearance over antipyrine clearance.

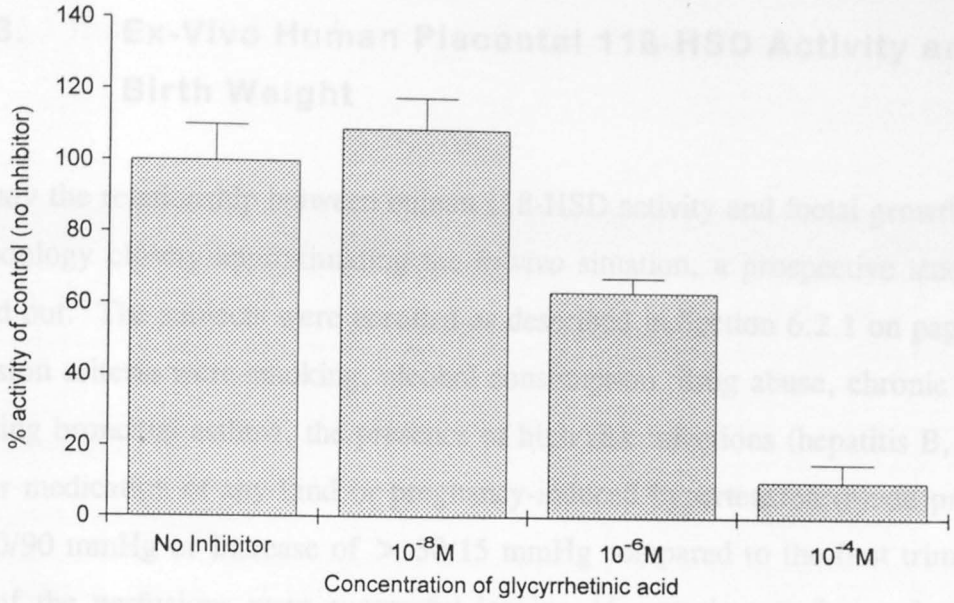


Figure 6.12 Inhibition of Human 11ß-HSD in the Perfused Placenta

The figure shows the potent inhibition of human placental 11ß-HSD *ex-vivo*, using the active lipid soluble constituent of liquorice, glycyrrhetinic acid. Columns represent means of 4 perfusions, bars are + SEM. No inhibitor is t = 40 - 70 min and then each glycyrrhetinic acid concentration was perfused for 30 min. IC_{50} is approximately 3 x 10^{-6} M.

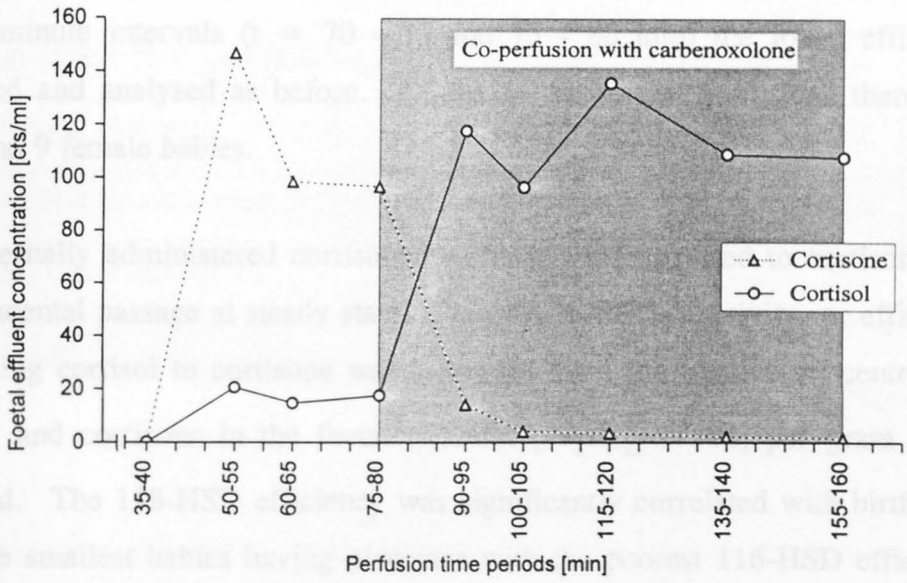


Figure 6.13 The Effect of Carbenoxolone on Placental 11ß-HSD *Ex-Vivo*

The first 40 minutes were washout as usual, followed by perfusion with 50 nM cortisol only from 40 - 80 min. Carbenoxolone was then added to the medium (10^{-4} M) from 80 - 160 min. Both lines indicate concentration of label appearing in the foetal effluent at the indicated time points. Cortisone generation from cortisol is completely inhibited by carbenoxolone.

6.3.3. Ex-Vivo Human Placental 11ß-HSD Activity and Birth Weight

To study the relationship between human 11ß-HSD activity and foetal growth using methodology closely approximating the *in-vivo* situation, a prospective study was carried out. The subjects were enrolled as described in Section 6.2.1 on page 141. Exclusion criteria were smoking, alcohol consumption, drug abuse, chronic illness including bronchial asthma, the presence of high risk infections (hepatitis B, HIV), regular medication of any kind or pregnancy-induced hypertension (blood pressure > 140/90 mmHg or increase of > 30/15 mmHg compared to the first trimester). 80% of the perfusions were successful leaving 16 experiments for analysis (see Section 6.2.1 for details).

The first 40 min were washout, followed by 40 min of perfusion with 50 nM cold cortisol and < 0.5% ^{3}H-cortisol tracer, (chosen to reflect estimates of high physiological "free" glucocorticoid levels [Meulenberg & Hofman 1990], and ^{14}C-antipyrine allowing calculation of relative corticosteroid clearance. For the two last 5 minute intervals (t = 70 - 75 and 75 - 80 min) the foetal effluent was collected and analysed as before. Of the 16 successful perfusions there were 7 male and 9 female babies.

Of maternally administered cortisol, 69 - 90% was converted to cortisone during transplacental passage at steady state. Placental 11ß-HSD activity, or efficiency in converting cortisol to cortisone was estimated from the relative concentrations of cortisol and cortisone in the foetal effluent: $[^{E}/_{F+E} \times 100]$ per gram of tissue perfused. The 11ß-HSD efficiency was significantly correlated with birth weight, with the smallest babies having placentas with the poorest 11ß-HSD efficiency ($n = 16$, $r = 0.67$, $r^2 = 0.45$, $p < 0.005$, Figure 6.14). A similar relationship was found between total placental 11ß-HSD efficiency (11ß-HSD efficiency per gram times total placental wet weight) and birth weight ($n = 16$, $r = 0.61$, $r^2 = 0.37$, $p < 0.02$; Figure 6.15).

Table 6.1 Human 11ß-HSD *Ex-Vivo*: Subject and Study Details

Variable	N	Mean	Range	SEM
Age at delivery [years]	16	27.3	19.8 - 34.1	1.0
Length of gestation [weeks]	16	40.4	38.4 - 41.7	0.3
Birth weight [kg]	16	3.6	3.1 - 4.1	0.1
Placental weight [g]	16	483	394 - 569	13
pO_2 step-up across foetal cotyledon [mmHg]	14	79	38 - 120	7
Time to start of foetal circulation [min]	16	14.8	11 - 21	0.8
Time to start of maternal circulation [min]	16	23.3	20 - 30	0.8
pH in foetal inflow	14	7.40	7.32 - 7.46	0.01
pH in foetal outflow	14	7.28	7.20 - 7.35	0.01
Foetal circuit temperature [°C]	14	37.2	36.6 - 37.7	0.1
Foetal output flow rate [ml/min]	16	6.1	5.3 - 6.6	0.1
Antipyrine clearance [ml/min]	16	3.3	0.9 - 8.2	0.5
Corticosteroid clearance (F + E) [ml/min]	16	2.5	1.0 - 4.3	0.3
Steroid Clearance Index	16	0.9	0.3 - 1.8	0.1

Conventionally, 11ß-HSD activity (efficiency) is calculated as % ratio of cortisone to total corticosteroids, but one could argue that the ability to generate cortisone would be just as good a measure of 11ß-HSD activity. Analysing the data in this way, the smallest offspring have placentas with the lowest ability to generate cortisone. For foetal effluent cortisone concentration (cts/ml) per g perfused

placenta: r = 0.53, p < 0.04. This did not reach statistical significance for total placenta (i.e. cts/ml/g times wet placental weight) although the same trend was seen: r = 0.46, p < 0.08. No correlation was found between placental 11ß-HSD efficiency and placental weight - one should note that 11ß-HSD activity is expressed per weight of placenta and thus placental weight is a major determinant in both the parameters being correlated.

At the beginning of each perfusion, samples from an adjacent cotyledon were immediately frozen at -70 °C for later 11ß-HSD assay *in-vitro*. There was no correlation between *in-vitro* activity of the frozen specimens and *ex-vivo* activity, and no correlation was found between *in-vitro* activity and either birth weight or placental weight, in keeping with the data presented in Section 5.

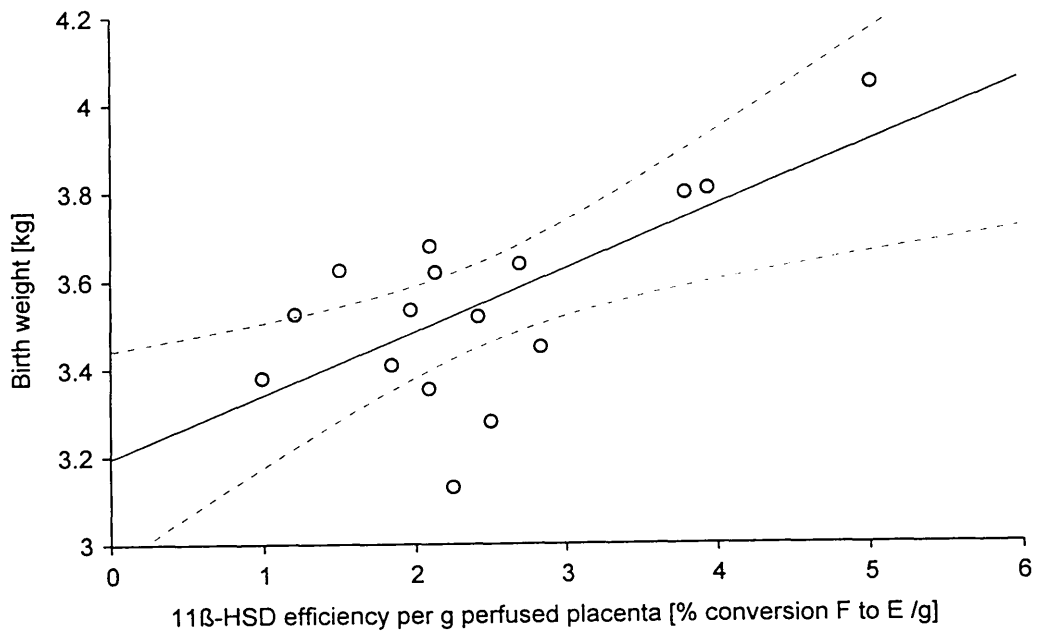


Figure 6.14 Human Birth Weight & Placental 11ß-HSD Efficiency *Ex-Vivo*: A

Human placental 11ß-HSD *ex-vivo* correlates highly significantly with birth weight. Pearson's r = 0.67, p < 0.005, n = 16. The solid line is the regression line with dotted lines outlining 95% confidence intervals for the regression. 11ß-HSD efficiency: $({}^{E}/_{E+F})$/g perfused placental tissue.

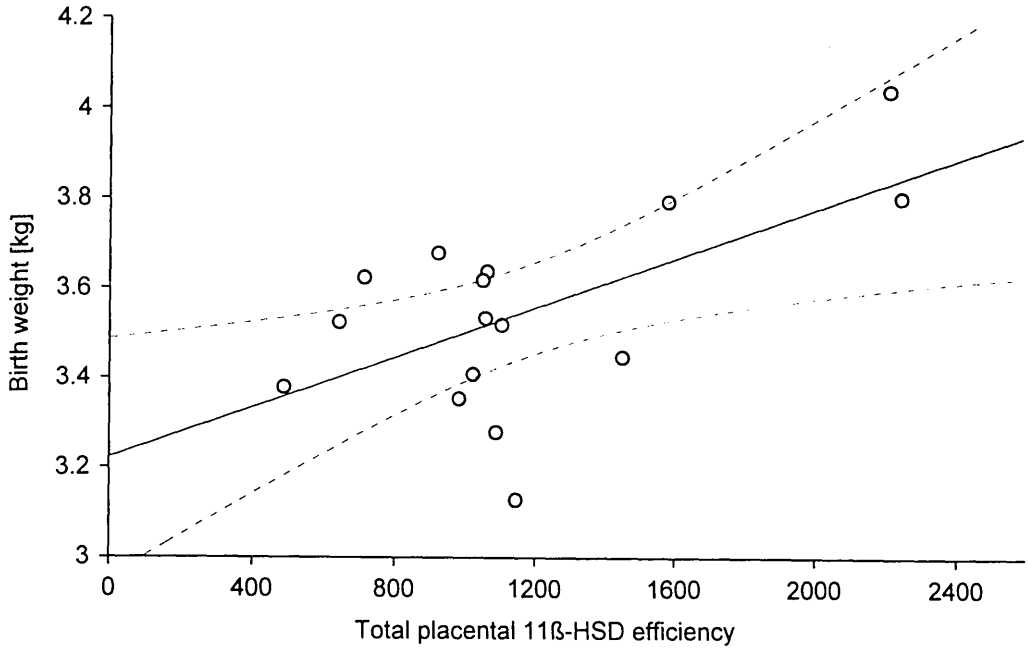


Figure 6.15 Human Birth Weight & Placental 11ß-HSD Efficiency *Ex-Vivo*: B

Total human placental 11ß-HSD *ex-vivo* correlates highly significantly with birth weight. Pearson's r = 0.61, $p < 0.02$, n = 16. The solid line is the regression line with dotted lines outlining 95% confidence intervals for the regression. Total placental 11ß-HSD efficiency is foetal effluent ($^{E}/_{E+F}$)/g perfused placental tissue times wet placental weight.

6.3.4. Human Placental 11ß-HSD Activity Ex-Vivo, Alcohol & Smoking

Various environmental influences are known to be important in determining foeto-placental growth. For example, life at high altitude (hypoxia) induces the interesting combination of low birth weight and high placental weight [Krüger & Arias-Stella 1970], as does maternal iron deficiency anaemia [Godfrey et al., 1991]. Streptozotocin-induced diabetes and ethanol produce similar effects in rats [Sybulski & Maughan 1971; Sanchis et al., 1986], and the growth retarding actions of smoking are well documented. The postnatal effects of prenatal ethanol exposure are modified by maternal adrenalectomy [Redei et al., 1993], and unpublished *in-vitro* data from our own laboratory has demonstrated a dose-dependent inhibition of liver 11ß-HSD by ethanol. Thus, although the precise causes are unknown, it is not unreasonable to propose that increased foetal

glucocorticoid exposure consequent upon attenuated placental 11ß-HSD may represent one possible unifying mechanism whereby environmental factors influence foeto-placental growth. Therefore, a series of perfusions were undertaken in order to look at the possibility of smoking, nicotine or ethanol affecting placental 11ß-HSD activity in the perfused human placenta.

First, three pregnant women were enrolled at term delivery according to the same criteria as detailed in Section 6.2.1, but this time they all admitted to smoking > 10 cigarettes daily through pregnancy. The placentas were perfused as before and 11ß-HSD efficiency compared with the 16 healthy placentas discussed in the previous Section. Second, healthy placentas from 6 non-smokers were perfused as previously described, maternal side only with washout/recovery from 0 - 40 min, 50 nM cortisol from 40 - 80 but then 50 nM cortisol + 0.2 μM nicotine from 80 - 125 min. This level of nicotine is equivalent to the plasma levels found in chronic heavy smokers [Russell et al., 1975]. Foetal effluent samples were collected over the last two 5 min periods of both the cortisol and the cortisol + nicotine phases, analysed separately as before and the data averaged for analysis. Third, in 5 separate perfusions, ethanol replaced nicotine in the concentration of 2 mg/ml, enough to intoxicate an average male. Data were processed as for nicotine. 11ß-HSD activity was expressed as $[(^{E}/_{F+E}) \times 100]$/g tissue perfused and similarly per total placental weight.

The mean 11ß-HSD efficiency (% conversion F to E - abbreviated as % in this Section) in the 6 placentas perfused in succession without and with nicotine was no different when nicotine was co-perfused with cortisol (t-test for dependent samples: pre-nicotine 11ß-HSD efficiency per g tissue perfused was 2.86 ± 0.59 $\%g^{-1}$ and with nicotine 2.81 ± 0.55 $\%g^{-1}$). The same applied per total placenta (1424 ± 283 % vs. 1393 ± 254 % with nicotine). The 3 smokers delivered non-significantly lighter babies than the normals (3.4 ± 0.1 kg vs. 3.6 ± 0.1 kg normals) and significantly heavier placentas (569 ± 61 g vs. 483 ± 13 g normals, $p < 0.04$: t-test). However, the efficiency of cortisol inactivation in the 3 smokers

was no different from the 16 controls (t-test for independent samples); 11ß-HSD efficiency per gram perfused tissue was 2.45 ± 0.26 $\%g^{-1}$ in smokers compared with 2.14 ± 0.17 $\%g^{-1}$ in controls. When calculated as total placental 11ß-HSD efficiency, the figures were 1195 ± 43 % in smokers and 1172 ± 123 % in normals.

Similarly, the dose of ethanol used (2 mg/ml) in perfused healthy placentas (n = 5) did not impair 11ß-HSD efficiency as estimated by t-test for dependent samples. Thus 11ß-HSD efficiency per g perfused tissue pre-ethanol was 2.35 ± 0.40 $\%g^{-1}$ vs. 2.33 ± 0.42 $\%g^{-1}$ with ethanol co-perfused. For total placental 11ß-HSD efficiency, this was also true, the activities for the 5 placentas being 1077 ± 141 % pre-ethanol vs. 1067 ± 152 % with ethanol co-perfused.

6.4. DISCUSSION

The validation experiments for the *ex-vivo*, dual circuit, isolated placental cotyledon perfusion methodology that was developed here, add confidence to the notion that this methodology gives results as close as possible to the *in-vivo* situation at the end of pregnancy, which clearly cannot be investigated *in-situ*. The start of foetal perfusion was within 15 min of delivery and perfusion for an extended period did not appear to compromise placental structural integrity, as assessed by both light- and electron-microscopy; sections taken from fresh placentas and after 3 h of perfusion were remarkably similar, indicating that few, if any, structural abnormalities were attributable to the perfusion itself. Several tissue samples were looked at, in all of which the morphology was identically well preserved. Therefore, the samples are likely to be representative of the whole cotyledon.

Further support for the viability of the preparation was obtained from the pattern of foetal effluent lactate release. Previous studies have shown a rapid fall in lactate

concentration in the foetal effluent of perfused cotyledons under oxygenated conditions during the first few minutes, levelling off in the following 15 minutes [Young & Schneider 1984]. This closely matches the experiments described here. If the preparation suffers hypoxia or anoxia there is no fall, and sometimes a rise in lactate concentration in the foetal effluent [Cannell et al., 1988; Illsley et al., 1984; Bloxam 1985]. The sensitivity of our assay was sufficient to detect the effects on lactate production of potential adverse conditions like hypoxia/anoxia. Illsley [Illsley et al., 1984] reports lactate production rate of ca. 175 μmol/min/kg under oxygenated conditions, rising by a factor of 1.5 - 2 under anoxia (recirculating foetal perfusate with the same flow rate as here). The sensitivity of our assay is 0.2 mM which approximately translates into minimum production rate detectable of 32 μmol/min/kg, (sampling volume 1.5 ml, flow rate 6 ml/min, using average cotyledon weight as 38 g, n = 16).

Not only is the ultrastructure adequately preserved, but so importantly is 11ß-HSD activity, which is relatively unstable in placental homogenates [Brown et al., 1993; Lakshmi et al., 1993], particularly if frozen as was observed here (Section 5). *Ex-vivo* 11ß-HSD activity was thus stable and preserved over an extended experimental period (Figure 6.3). The fact that there was no difference in 11ß-HSD activity estimated from endogenous steroid levels (Figure 6.3) and the activity calculated from data based on artificial perfusion (pre 40 min compared with post 40 min), further supports the notion that the data obtained using this method parallel closely metabolic capacities *in-vivo*. It was therefore of interest that there seemed to be a certain amount of cortisol leakage (bypassing metabolism) in some perfusions during the first 15 min of cortisol perfusion (t = 40 - 55 min, Figure 6.6). An "isotope effect" as an explanation for this is not likely as this leakage was not apparent in all the perfusions. This supports the use of an extended recovery period (up to 55 min; all 11ß-HSD activities analysed here are from later times) and the use only of data from steady state for analysis.

It is noteworthy that there seemed to be a marked materno-foetal gradient across the placenta for total corticosteroids, suggesting that 11ß-HSD wasn't the only mechanism controlling access of glucocorticoids to the foetus. Again, an "isotope" effect can be suggested but is not likely as this gradient was also observed while perfusing with cold steroids only. This could also be attributable to a poor overlap of the maternal and foetal circulations in these experiments (supported by a gradient for antipyrine) or due to sequestration of cortisol in placental tissue, the latter notion given support by the observation from Figure 6.10, that the fitted line does not intercept the maternal axis through zero - thus a certain minimal maternal concentration is probably necessary for any transplacental steroid passage to take place. If the materno-foetal gradient of steroid was only due to perfusion inequalities (poor materno-foetal circulation overlap), this should affect antipyrine equally to cortisol and the effect of changing maternal concentrations should not affect relative steroid clearance (relative to antipyrine). In fact the relative clearance did increase with increasing maternal concentrations, as shown in Figure 6.11. Therefore factors like lipid solubility are likely to be contributing to the placental glucocorticoid barrier.

The fact that the total amount of steroids transferred across the placenta from the maternal to the foetal side, increased with increasing maternal concentrations is important as it means that increasing maternal concentration will result in higher absolute foetal concentrations of cortisol, although the same proportion is metabolised to cortisone at all maternal concentrations.

An important fact however is that the activity of 11ß-HSD remains constant over a wide range of maternal glucocorticoid concentrations, from low to high physiological levels. The majority of transplacentally passing corticosteroids are inactivated by 11ß-HSD (here at 50 nM: 69 - 90%), no matter what the absolute concentration. Therefore, a defect in 11ß-HSD would presumably be of far greater consequence than any variations in maternal cortisol concentrations within the

physiological range. Thus intact placenta has a high capacity to inactivate maternal glucocorticoids, but whether the capacity of the enzyme can be flooded by greatly elevated (> 200 nM) free maternal cortisol levels (in stress or disease) remains unknown. Another way of controlling the total transplacental steroid passage would be to add proteins to the maternal perfusate. This would lower the absolute total corticosteroid transfer but apparently does not affect 11ß-HSD activity [Dancis et al., 1978].

Emphasising the role of 11ß-HSD as the main player in the barrier to maternal glucocorticoids are the studies with carbenoxolone and glycyrrhetinic acid. Thus by inhibiting 11ß-HSD there is free transplacental passage of maternal cortisol. Previous studies with the synthetic glucocorticoid prednisolone and cortisol [Addison et al., 1991; Dodds et al., 1993], which used recirculating maternal and foetal perfusates, pharmacological free steroid concentrations (μM range), found evidence of metabolism by 5ß-reductase and 20α/ß-hydroxysteroid dehydrogenase. These studies are likely to be confounded by i) the use of high steroid concentrations, resulting in metabolism by normally quiescent or low affinity enzymes, and ii) the practice of recirculating perfusates which will subject cortisone (or prednisone) to further metabolism, the products thus wrongly believed to be the result of primary cortisol metabolism. This was later confirmed in studies with prednisolone, where no products were detected if glycyrrhetinic acid was included in the perfusate [Addison et al., 1993]. The studies here found no significant contribution of any other enzyme than 11ß-HSD to placental cortisol metabolism when using physiological concentrations of cortisol and non-recirculating perfusions - thus 11ß-HSD is the major contributor to the human placental glucocorticoid barrier.

The prospective study on birth weight and placental 11ß-HSD activity *ex-vivo*, shows as in the validation studies, that 11ß-HSD potently inactivates maternal glucocorticoids in intact human placental tissue at term and importantly, that the

efficiency of this barrier varies considerably, correlating with birth weight. These data in conjunction with studies on the effects of treatment of animals with glucocorticoids [Benediktsson et al., 1993] and 11ß-HSD inhibitors [Lindsay et al., 1994b] therefore lend support the hypothesis [Edwards et al., 1993a] that excessive exposure of the foetus to maternal glucocorticoids due to deficient placental 11ß-HSD retards intrauterine growth in humans.

Using the *ex-vivo* system it was found that more than two thirds of maternally-applied cortisol was converted to inert cortisone during placental passage. This activity was due to 11ß-HSD (as discussed above), as cortisone is the only product detected and cortisol inactivation was prevented by glycyrrhetinic acid, a potent inhibitor of the enzyme, particularly in the 11ß-dehydrogenase (cortisol inactivating) direction [Stewart et al., 1990b]. Previous human studies *in-vitro* [Brown et al., 1993; Blasco et al., 1986], *ex-vivo* (as here) at term [Dancis et al., 1978], and *in-vivo* at mid-gestation [Murphy et al., 1974; Pasqualini et al., 1970] and at term [Beitins et al., 1973] have demonstrated that 11ß-dehydrogenation (steroid inactivation) is the predominant activity of placental 11ß-HSD. This presumably reflects the absence of reversible 11ß-HSD-1 in purified human placental preparations [Brown et al., 1993]. Placental 11ß-HSD showed similar fractional cortisol metabolism over a range of maternal steroid concentrations, which probably span the broad variation of concentrations of "free" cortisol reaching the placenta via the uterine artery, as Blasco had previously calculated from *in-vitro* experiments [Blasco et al., 1986].

However, the exclusion of maternal cortisol from the foetus was not complete confirming previous estimates of a minor, but detectable, contribution of maternal glucocorticoid to foetal plasma cortisol [Murphy et al., 1974; Pasqualini et al., 1970; Dancis et al., 1978]. Clearly, within a certain tolerance, feedback of transplacentally-passed cortisol upon the foetal hypothalamic-pituitary-adrenal axis will reduce foetal adrenal secretion and maintain foetal cortisol levels.

Nevertheless, given that cortisol levels in the foetus are several fold lower than in the maternal circulation [Campbell & Murphy 1977], a relative defect in placental 11ß-HSD is likely to have far greater consequences in terms of the overall foetal glucocorticoid load, than any alterations in foetal adrenal cortisol production per se.

The activity of 11ß-HSD in intact placenta showed considerable variation, either expressed per gram of placental tissue or per whole placenta (presumably a better estimate of the total amount of maternal cortisol reaching the foetal circulation). The mechanism(s) producing this variation (genetic and/or environmental) remain to be determined in humans, but studies in baboons suggest the importance of foetal and placental hormones, notably sex steroids [Pepe et al., 1988]. We have attempted to exclude some maternal factors (see exclusion criteria), but the role of maternal nutrition has not been examined and there is some evidence that this may affect placental 11ß-HSD in rats [Phillips et al., 1994].

From the studies presented here on nicotine and ethanol, it appears that neither agent acutely affects the ability of placental 11ß-HSD to inactivate glucocorticoids. Similarly, the efficiency of placental 11ß-HSD does not seem to be altered in smokers as compared with non-smokers. There are though a few points that deserve to be mentioned: i) the time period of ethanol and nicotine perfusions was only 45 min and therefore chronic effects of these agents on placental 11ß-HSD have not been excluded, and ii) only 3 smokers were included in the study, which may therefore not have had the power to detect a small difference in placental 11ß-HSD activity between smokers and non-smokers, and iii) if the growth retarding effects of smoking are via interaction with placental 11ß-HSD, that is probably via other constituents of cigarette smoke than nicotine, and finally iv) the reported smoking habits in smokers and controls were not confirmed objectively. These agents therefore deserve further investigation using different experimental models, for example cultured trophoblast cells.

The close direct correlation of 11ß-HSD activity in intact cotyledons with birth weight, confirms similar observations with fresh placental homogenates in rats [Benediktsson et al., 1993]. That this relationship with birth weight might be causal is suggested by data showing that administration of 11ß-HSD inhibitors to pregnant rats reduces birth weight [Lindsay et al., 1994b], an action dependent upon intact maternal adrenal glands (unpublished observations). Moreover, blood pressure in the adult offspring was elevated. Whether placental 11ß-HSD deficiency correlates with elevated blood pressure in humans in later life remains to be determined, but the data presented here lend support to the hypothesis that deficiency of placental metabolism of maternal glucocorticoids may provide a mechanism to explain the epidemiological link between low birth weight and subsequent common disorders of adulthood.

7. HUMAN FOETAL OSTEOCALCIN AND PLACENTAL 11ß-HSD

7.1. INTRODUCTION

Foetal growth is affected by numerous influences including glucocorticoids [Reinisch et al., 1978; Benediktsson et al., 1993] which do not only impair growth but also permanently affect differentiation and maturation of specific target organs in animals and humans [Slotkin et al., 1992b; Slotkin et al., 1992a; Ballard 1979]. A few authors have reviewed the data on outcome of babies exposed transiently (for approximately 24 h) to glucocorticoids (as is becoming more and more common all over the world as therapy to induce pulmonary surfactant production, in cases of threatened premature labour). There is little information available on birth weight or other short term parameters, or even longer term effects, and most authors have failed to find any detrimental effects of prenatal brief dexamethasone exposure in humans [Taeusch, Jr. 1975; Sidhu 1987]. However, some evidence exists for altered organ development in animals in this setting [Epstein et al., 1977; Johnson et al., 1981]. We also know that treatment with dexamethasone for a few days can alter development of specific target organs in animals, as mentioned above, and that chronic low doses are able not only to alter foetal growth, but also directly elevate blood pressure *in-utero* in sheep, possibly via altered vascular sensitivity to angiotensin-II [Tangalakis et al., 1992]. Recent data also show that in animals, glucocorticoids permanently programme higher blood pressures in later life [Benediktsson et al., 1993].

There are two potential sources of glucocorticoids in the foetal circulation; the foetal adrenal, which secretes cortisol from first trimester [Murphy 1973; Beitins et al., 1973], and transplacental passage of maternal corticosteroids. As the maternal plasma levels are 2 - 10 times higher than foetal concentrations [Campbell &

Murphy 1977], the integrity of the placental glucocorticoid barrier is critical. The previous Section (Section 6) in this thesis has confirmed earlier suggestions [Murphy et al., 1974; Dancis et al., 1978; Beitins et al., 1973; Dormer & France 1973] that this barrier in humans is placental 11ß-HSD, the efficiency of which varies considerably in both humans (see 6.3.3) and animals [Benediktsson et al., 1993].

Moreover, human placental 11ß-HSD is the only significant mechanism contributing to the placental glucocorticoid barrier (Section 6) protecting the human foetus from maternal glucocorticoids throughout pregnancy as documented in several previous studies: *in-vitro* [Brown et al., 1993; Blasco et al., 1986], *ex-vivo* (Section 6) at term [Dancis et al., 1978], and *in-vivo* at mid-gestation [Murphy et al., 1974; Pasqualini et al., 1970] and at term [Beitins et al., 1973]. Also, there is no change in human placental 11ß-HSD activity in the short term during labour [López Bernal et al., 1982b]. This presumably reflects the absence of reversible 11ß-HSD-1 in purified human placental preparations [Brown et al., 1993]. This is in marked contrast to what is believed to be the case in some other species, where the prevailing direction of enzyme activity changes during pregnancy, favouring active steroid (reduction cortisone converted to cortisol) at mid-gestation but at term the reverse (dehydrogenation inactivating cortisol) [Pepe & Albrecht 1984b].

As is clear from the data presented here previously (Section 6) on human placental 11ß-HSD, the activity can readily be determined with *ex-vivo* placental perfusion which probably gives a true estimation of *in-vivo* efficiency. The method used (*ex-vivo* perfusion) is however obviously not suited for mass studies, and as alluded to previously, *in-vitro* experiments are hampered by storage problems. Furthermore, no studies exist relating the effectiveness of placental glucocorticoid inactivation *in-vivo* with actual foetal glucocorticoid exposure. It would thus aid further research considerably if a serum marker of *in-vivo* placental 11ß-HSD activity could be found, which we knew reflected the effects of glucocorticoids. At first glance, cord blood cortisol levels are attractive.

Maternal total cortisol levels rise during pregnancy, as does the free biologically active fraction [Meulenberg & Hofman 1990; DemeyPonsart et al., 1982; Scott et al., 1990]. The levels remain constant after 26 weeks until labour [Carr et al., 1981], but at term rise markedly in both the mother and the foetus (for a review, see Murphy 1983), depending for example on mode of delivery [Leong & Murphy 1976]. The intrinsic variation of both term maternal and cord blood cortisol levels thus poses a problem and it is clear that term cord cortisol may not be representative of "usual" cord levels (the usual level of foetal cortisol exposure). One would have to look at surrogate markers to get an idea about long term cortisol exposure, reflecting the function of 11ß-HSD.

Glucocorticoids inhibit activity of mature osteoblasts (and bone formation) but promote differentiation of marrow osteoprogenitor cells although their proliferation is prevented [Cheng et al., 1994]. Osteocalcin (Bone GLA-protein or BGP) is a small non-collagenous protein, specific for bone tissue and dentine [Delmas 1990]. Osteocalcin is synthesised predominantly by osteoblasts and a small fraction of the protein is released into the circulation. Chronic glucocorticoid excess depresses circulating osteocalcin levels, the effect becoming apparent after several days [Delmas 1990]. Osteocalcin levels are indeed a very sensitive marker of corticosteroid exposure, blood levels in adults being reduced after inhaling only 400 μg of the synthetic glucocorticoid beclomethasone dipropionate daily for 5 days [Teelucksingh et al., 1991b]. The relationship between osteocalcin in cord blood and derived indices of 11ß-HSD was therefore examined.

7.2. METHODS

7.2.1. Subject Selection and Sampling

Twenty one consecutive women were enrolled on admission for delivery at the Simpson Memorial Maternity Pavilion in Edinburgh - Dr. J. Brennand collected

the samples. Two were excluded because of coincident disorders (gestational diabetes, type I diabetes mellitus) and in two further cases the plasma samples obtained were insufficient for assay. The time and mode of delivery varied and the subjects were a mixture of primi- and multi-parous women. The umbilical cord was kept clamped through the third stage of delivery, at the end of which blood was collected separately from the artery and vein into lithium-heparin tubes. Following centrifugation, plasma was frozen at -20 °C within 1 h and stored until assayed.

7.2.2. Assays and Data Analysis

Cortisol, cortisone and osteocalcin were measured by in-house second antibody radioimmunoassays as described in Section 2.3.

Differences between means of continuous variables were tested using analysis of variance (ANOVA). Pearson's product moment correlation was used to seek associations between osteocalcin levels and indices of placental 11ß-HSD activity. Several indices were used for determining the effect of placental 11ß-HSD activity on cord blood osteocalcin levels. Firstly, cord venous plasma cortisone (the product of the reaction catalysed by 11ß-HSD). Secondly, overall 11ß-HSD activity, as conventionally assessed, by the percentage cortisone of total glucocorticoids ($^{E}/_{(F+E)}$) in cord venous blood. However, although most cortisone in the cord blood will derive from placental metabolism of maternal cortisol [Beitins et al., 1973], some will be derived from the foetus, which has endogenous 11ß-HSD [Murphy 1981; Stewart et al., 1994a]. We therefore also determined an 11ß-HSD Activity Index to account for this foetal (cord arterial) component of glucocorticoids, calculated as [cord venous ($^{E}/_{(F+E)}$)] minus [cord arterial ($^{E}/_{(F+E)}$)]. A high ratio of cortisone to total glucocorticoid (F+E) indicates high cortisol inactivating function of placental 11ß-HSD and the latter

half of the 11ß-HSD Activity Index equation (arterial steroid concentrations) corrects for the foetal contribution.

7.3. RESULTS

Cortisol levels were significantly higher in the cord artery (241.6 ± 25.7 nM) than the vein (163.1 ± 13.4 nM; $p < 0.0005$), but no difference was observed for mean cortisone levels (umbilical artery 26.9 ± 4.1 nM; vein 29.5 ± 4.4 nM) or osteocalcin levels (artery 25.4 ± 1.8 µg/l; vein 29.2 ± 2.8 µg/l; mean 27.3 ± 2.1 µg/l). Since there was no difference between arterial (blood travelling from foetus) and venous osteocalcin levels (osteocalcin in the foetal circulation does not reflect maternal levels but is derived entirely from the foetus [Delmas et al., 1987; Shima et al., 1985]), mean cord osteocalcin levels were used for all correlation analyses.

Venous cord cortisone and mean cord osteocalcin were significantly and positively correlated ($r = 0.57$, $p < 0.02$). The ratio of cortisone to total steroids in cord vein plasma ($^{E}/_{(F+E)}$) correlated significantly with mean cord osteocalcin ($r = 0.57$; $p < 0.02$). A significant positive correlation ($r = 0.58$; $p < 0.02$) was found between the cord 11ß-HSD Activity Index and mean cord osteocalcin levels (Figure 7.2). Venous cord cortisol did not show a significant correlation with cord osteocalcin.

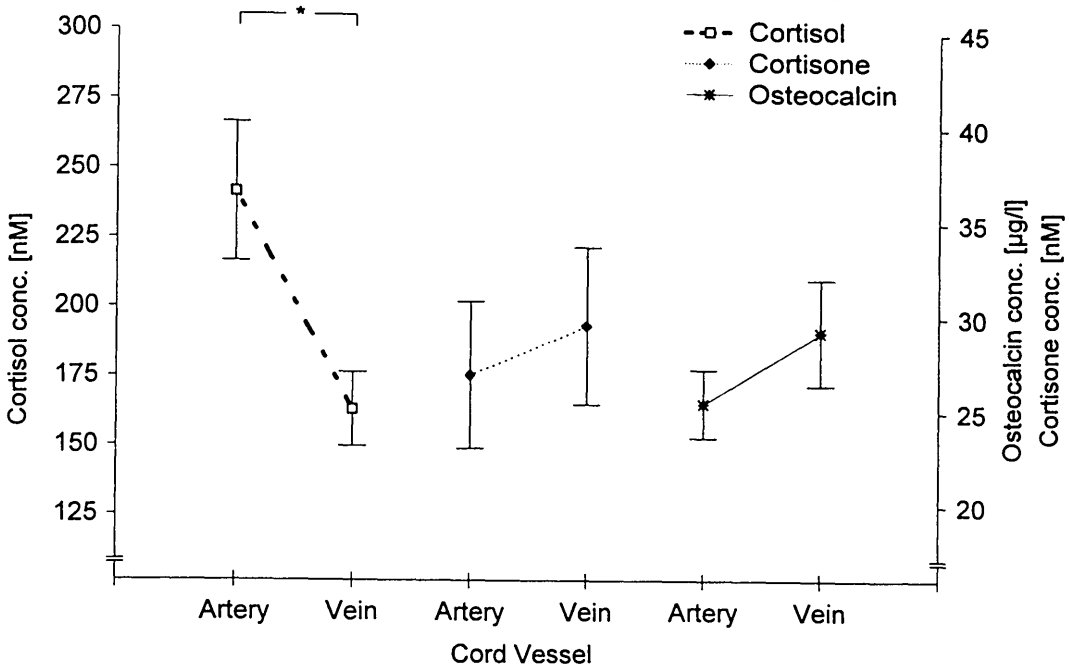


Figure 7.1 Term Cord Blood Steroid and Osteocalcin Levels

The figure shows mean ± SEM for cortisol, cortisone and osteocalcin in cord arterial and venous blood at term. *: $p < 0.0005$ with ANOVA.

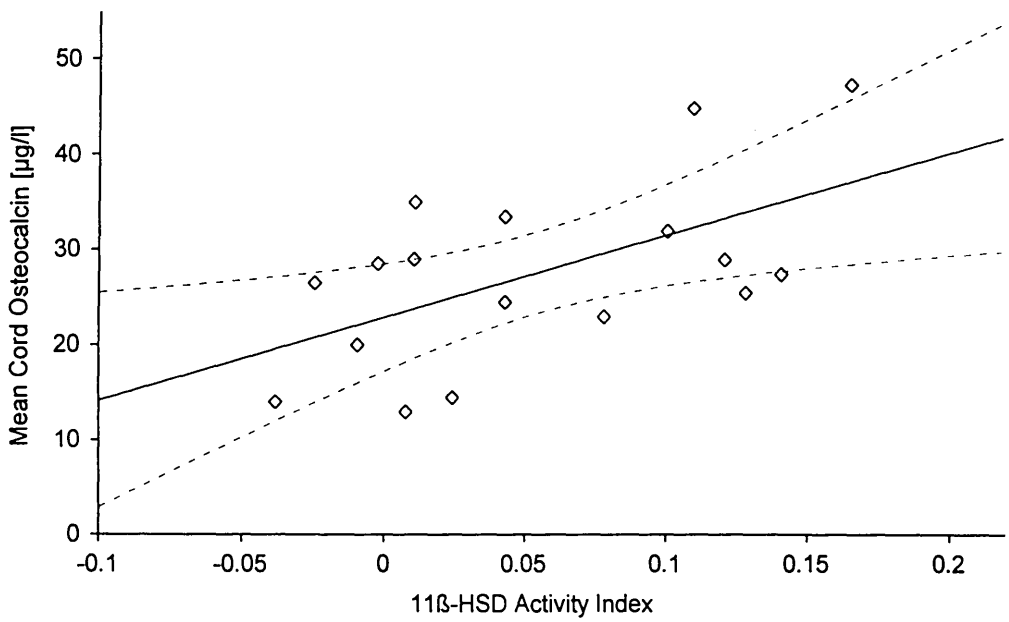


Figure 7.2 Mean Cord Osteocalcin and 11ß-HSD Activity Index

The relationship between placental 11ß-HSD Activity Index on the horizontal axis and mean cord osteocalcin levels on the Y-axis. The solid line indicates the regression line while the dotted lines indicate the 95% confidence interval for the regression ($r = 0.58$, $r^2 = 0.33$; $p < 0.02$; $n = 17$).

7.4. DISCUSSION

Cord steroid values have been the subject of a number of studies reviewed by Murphy [Murphy 1983]. In summary, these studies have used variable methodologies to arrive at different results, cortisol levels ranging from 90 - 1200 nM. Murphy [Murphy 1983] considers the probable true mean value to be approximately 180 - 250 nM for pooled cord blood, based on rigorous scrutiny of the methodology of the studies concerned. The values obtained in this study compare favourably with this summary (artery 242 nM and vein 163 nM). As regards the difference between arterial and venous values, equal number of studies have reported the venous cortisol higher than the arterial and vice versa. It is probable that the artery carrying blood from the foetus has higher values as was shown in the only study which has employed foetoscopy and thus obtained blood from the undisturbed foetus [Partsch et al., 1991], although the variation in this study was also great, possibly reflecting the natural variation in placental 11ß-HSD effectiveness. As regards cortisone levels, three studies have looked at arterial and venous cord levels at mid-gestation [Murphy 1973; Murphy 1977a; Partsch et al., 1991], arriving at similar values as presented here with no difference between vein and artery. Murphy however did look at term cortisone levels with and without labour [Murphy 1977a] and found non-significantly higher levels in the umbilical vein (blood travelling to the foetus), as here. Murphy however, found the levels during labour to be approximately twice the levels when no labour was experienced during elective caesarean section. The samples here are a mixture of labour/no labour.

Cord blood osteocalcin levels which have also been the subject of a few studies appear, at least in substantial part, to reflect foetal production [Delmas et al., 1987; Shima et al., 1985]. This contention is supported by cord levels being at least 2 - 3 times higher than those in the maternal blood [Delmas et al., 1987; Seki et al., 1993; Shima et al., 1985], although the levels reported in individual studies vary considerably. Of these studies, only one looked at venous levels [Delmas et

al., 1987], reporting levels half the levels observed here (29 μg/l). Another [Seki et al., 1993] looked also at arterial levels and found them to be approximately 4 μg/l while venous was reported as ca. 10 μg/l, again lower than mine (here 25 μg/l). Two other studies in contrast reported levels higher than documented here, mixed cord blood levels being approximately 40 - 55 μg/l (mean here 27 μg/l). The reason for this apparent discordance of different osteocalcin assays is unknown, and has recently been the subject of a publication where the differences were found to be most marked in certain categories of patients (renal failure where different osteocalcin fragments are likely to be found) [Masters et al., 1994]. Thus different assays are probably not directly comparable.

The hypothesis, that human placental 11ß-HSD activity *in-vivo* crucially determines foetal glucocorticoid exposure over and above any contribution from the foetal adrenal, has here been addressed in three different, albeit related ways. Firstly by examining cord venous cortisone levels, secondly by looking at the ratio of cortisone to total glucocorticoids in cord venous blood and thirdly by looking at 11ß-HSD Activity Index, which corrects placental glucocorticoid metabolism for the recirculating and/or foetal contribution. All indices correlated significantly and positively with cord osteocalcin levels, in keeping with the notion that glucocorticoids depress plasma osteocalcin levels even in foetal life [Delmas et al., 1987; Teelucksingh et al., 1991b]. A significant correlation of venous cord cortisol (blood travelling towards the foetus) and cord osteocalcin was not found, which was not unexpected as cortisol concentrations are notoriously variable, particularly with the acute stresses of parturition.

At term, approximately 90% of cord blood cortisone is of maternal origin (via placental metabolism of maternal cortisol) [Beitins et al., 1973]. Cord cortisone is likely to be a better indicator of overall placental 11ß-HSD activity than cord cortisol, as cortisone shows little acute response to stress or circadian influences [Walker et al., 1992a]. Higher cortisone levels in cord venous blood therefore indicate more efficient placental inactivation of maternal cortisol, a notion supported by the close relationship between cord venous cortisone levels and the

11ß-HSD Activity Index. Moreover, since the levels of active glucocorticoids in the foetal circulation are several fold lower [Campbell & Murphy 1977] than those in the maternal circulation, a relative defect in the placental glucocorticoid barrier (11ß-HSD) has far greater potential consequences in terms of the foetal glucocorticoid load, than any alterations in foetal adrenal cortisol production.

The functional importance of cord blood osteocalcin is unknown, but may, by analogy with children and adults, reflect foetal osteoblast activity and hence bone formation and turnover [Delmas 1990]. Human osteoblasts express glucocorticoid receptors [Subramaniam et al., 1992]. Glucocorticoids promote the differentiation of human foetal and adult bone, particularly osteoblasts [Cheng et al., 1994; Shalhoub et al., 1992], but the apparent paradox is that they inhibit osteoprogenitor cell proliferation and also osteocalcin expression from differentiated osteoblasts [Cheng et al., 1994]. The latter effect is mediated by glucocorticoid receptor interactions with "cis-acting" sequences in the human osteocalcin gene promoter, which inhibit transcription, and by interference with vitamin D and/or growth factor-mediated stimulation of osteocalcin synthesis [Morrison & Eisman 1993]. Although the detailed molecular mechanisms require further study, it is intriguing to speculate that glucocorticoid exposure *in-utero* may programme adult bone parameters, perhaps by determining osteoblast numbers [Cheng et al., 1994]. Nevertheless, the estimation of cord blood osteocalcin will provide an important tool in future studies to determine whether excessive exposure to maternal glucocorticoids *in-utero* increases the risk of the development of hypertension, ischaemic heart disease and perhaps osteoporosis in later life in humans.

8. RÉSUMÉ

The research presented in this thesis was prompted by a growing body of evidence implicating events before or around birth in the pathogenesis of ischaemic heart disease. Those data originate mostly from this country, from the MRC Epidemiology Unit in Southampton [Barker 1992a], although similar observations have also been made in other countries. Not only is death from ischaemic heart disease predictable from parameters of early development like birth weight, but so is the development of several of the risk factors for this disease, which still is responsible for up to 50% of deaths in industrialised countries. In standard textbooks of medicine, hypertension is cited as one of 3 major risk factors for ischaemic heart disease, the other 2 being smoking and high cholesterol. Low birth weight however, has been calculated to be 3 times as strong a predictor for the occurrence of ischaemic heart disease as smoking. For hypertension, we are still unaware of the precise aetiology in up to 95% of identified adult cases.

Blood pressure levels track from infancy to adulthood, i.e. those individuals with the highest childhood blood pressures continue to have the highest blood pressures as adults, the pattern only being temporarily perturbed during the adolescent growth spurt [Lever & Harrap 1992]. This tracking of blood pressure levels is a strong argument for the notion that hypertension is programmed in early life. Indeed, in several discrete populations, low birth weight (sometimes in conjunction with increased placental weight) has been shown to potently predict high blood pressure levels, independently of adult "life-style" factors such as alcohol intake, smoking, obesity and social class.

Importantly, this correlation is not only observed at the extremes of the birth weight distribution, but is continuous throughout the normal range [Barker et al., 1990], and is observed already in infancy [Law et al., 1993], the association

growing stronger with the age of the population studied. Thus for each kg increase in birth weight, systolic blood pressure decreased by 5.2 mmHg in a population aged 64 - 71 years [Law et al., 1993], emphasising the strength of this association.

Many researchers believe that this association can be explained by maternal malnutrition leading to disturbed foetal development [Barker et al., 1993a]. We have been exploring the alternative hypothesis that glucocorticoid excess *in-utero* might play a role [Edwards et al., 1993a]. The latter hypothesis is supported by several lines of evidence:

1. Prenatal glucocorticoid excess retards foetal growth in both humans and animals [Reinisch et al., 1978; Benediktsson et al., 1993; Katz et al., 1990].

2. Glucocorticoids, like other steroids, during windows of development permanently programme biochemistry, tissue maturation and responses [Gustafsson & Stenberg 1974b; Meaney et al., 1993; Bian et al., 1992; Bian et al., 1993].

3. An example of the above is the foetal growth retardation and programming of offspring hypertension by dexamethasone treatment of pregnant rats [Benediktsson et al., 1993], dexamethasone largely escaping metabolism by the placental glucocorticoid barrier - 11ß-HSD.

4. Treatment of pregnant rats with inhibitors of 11ß-HSD also leads to low birth weight, offspring insulin resistance and hypertension, effects which are prevented by maternal adrenalectomy [Lindsay et al., 1994b; Lindsay et al., 1994a; Lindsay & Seckl 1994].

Therefore, 11ß-HSD is likely to be of key importance. This was addressed in the experiments presented here, by trying to answer the questions detailed in Section 1.6. The third Section (Rat Ovarian 11ß-HSD, page 88) deals with the possibility

of 11ß-HSD playing a role in controlling glucocorticoid exposure pre-implantation. Evidence for the presence of both 11ß-HSD-1 and 11ß-HSD-2 in the rat ovary was found. Thus ample NAD dependent 11ß-dehydrogenation activity (11ß-HSD-2) was found in homogenised ovaries, but in addition, northern blotting and *in-situ* hybridisation detected 11ß-HSD-1 mRNA expression, and immunohistochemistry confirmed the presence of 11ß-HSD-1 protein. There precise role of ovarian 11ß-HSD however still remains open to question. A role for ovarian 11ß-HSD in modulating the effects of cortisol on gonadotrophin induced steroidogenesis [Michael et al., 1993b] has been suggested, and also a potential role in determining the success of *in-vitro* fertilisation and embryo transfer [Michael et al., 1993a]. It is however by no means clear how the two 11ß-HSD isoforms interrelate in the ovary. Do they exist in the same cells? How are they regulated developmentally - is follicle maturation important? How does oocyte 11ß-HSD relate to 11ß-HSD in granulosa-lutein cells? Are glucocorticoids the substrate? These points all remain to be answered but a current priority in our laboratory is to examine the pattern of expression of 11ß-HSD-2, since the human cDNA is now available.

The fourth, fifth and sixth Sections address three principal questions. First, is placental 11ß-HSD the glucocorticoid barrier? Second, is there a relationship between the efficiency of glucocorticoid inactivation by placental 11ß-HSD and foetal growth? Third, are there species specific differences in this regard? These questions were addressed *in-vitro* and *ex-vivo* in humans and *in-vitro* in an animal model, the rat, which has comparable placental anatomy. The presence of 11ß-HSD bioactivity, being dominantly 11ß-dehydrogenation, was confirmed *in-vitro* in both human and rat placentas.

In the rat, the presence of 11ß-HSD-1 mRNA and protein was also confirmed (Section 4, Rat Placental 11ß-HSD, page 101). A considerable natural variation of the activity of 11ß-HSD was observed and the intra-placental distribution of bioactivity was appropriate for the proposed barrier function. The activity of 11ß-

HSD per gram of placental homogenate was highly significantly correlated with both foetal growth ($r = 0.46$, $p < 0.0005$) and placental growth ($r = -0.64$, $p < 0.00001$), such that the smallest foetuses which also had the largest placentas, had the lowest placental enzyme activity (were therefore presumably exposed to the greatest amounts of maternal glucocorticoids). This pattern is strongly predictive of hypertension in some human epidemiological studies [Barker et al., 1990]. No reductase activity was found, although tissue manipulation may have destroyed that activity [Brown et al., 1993; Lakshmi et al., 1993]. Also, these studies can be criticised for being conducted *in-vitro*, in a non-human model. Further, remembering the storage problems with 11ß-HSD, one cannot exclude the presence of active 11ß-HSD-1 (mRNA and protein present), possibly promoting the generation of active glucocorticoids. Also in the baboon, 11ß-HSD seems to furnish the foetus with active glucocorticoids at mid-gestation (reductase), but at term functioning as an 11ß-dehydrogenase [Pepe & Albrecht 1985]. These data are not necessarily contradictory, as glucocorticoids may be needed to promote tissue/cell specific differentiation during windows of development, but detrimental at different windows of development.

In humans, both *in-vitro* and *ex-vivo* methodologies were employed. The lack of correlation between foetal growth (birth weight) and term placental 11ß-HSD activity *in-vitro* was disappointing (Section 5, Human Placental 11ß-HSD In-Vitro, page 122). However, the human placental enzyme is notoriously unstable *in-vitro* [Brown et al., 1993; Lakshmi et al., 1993]. The result was therefore not entirely surprising and further analysis of the data revealed an interesting pattern. Thus the oldest group of samples, which had been stored at -70 °C for 15 months, showed considerably less variation in activity than younger sample groups. This probably means that part of human 11ß-HSD is unstable under the storage conditions, making it impossible to draw any conclusions regarding placental 11ß-HSD and

foetal growth from stored samples. Again, any *in-vitro* result may not represent *in-vivo* physiology.

It was therefore necessary to resort to a different method, as closely paralleling human *in-vivo* physiology as possible - *in-vivo* experiments were clearly not feasible (Section 6, Human Placental 11ß-HSD Ex-Vivo, page 139). The *ex-vivo* dual circuit placental cotyledon perfusion was started on average within 15 minutes of delivery. All the parameters employed for assessing integrity and viability of the preparation (morphology by light and electron microscopy, metabolic stability over an extended time period, lactate production, volume conservation, circuit pressure, perfusate pO_2 and pH), indicated satisfactory function throughout the experimental period. The relative corticosteroid clearance was comparable to previously reported values, adding to confidence in the results. There was, as in the rat, a considerable variation in the efficiency of glucocorticoid inactivation by 11ß-HSD. However, the majority (69 - 90%) of maternally administered cortisol was metabolised to inert cortisone on transplacental passage.

The placental glucocorticoid barrier was abolished by classical 11ß-HSD inhibitors (glycyrrhetinic acid or carbenoxolone) - maternally administered cortisol passing unmetabolised through to the foetal circulation. Finally, and importantly, the ability of human placental 11ß-HSD to inactivate maternally administered cortisol, correlated positively and significantly with birth weight, either calculated per gram of perfused placental tissue ($r = 0.67$, $p < 0.005$), or per total placenta ($r = 0.61$, $p < 0.02$).

These studies can be criticised, i) for not looking at reductase activity *ex-vivo*, with reference to the baboon (see above), ii) only looking at term placentas, again with reference to the baboon placenta, iii) not doing parallel studies on other "reference" enzymes and iv) the correlations may not represent a cause and effect relationship. Regarding the two first points, the available data for human placentas

does not support the idea of reductase (11ß-HSD-1) being present, as assessed by *in-vivo* studies at mid-gestation [Murphy et al., 1974; Pasqualini et al., 1970], and *in-vitro* and *ex-vivo* studies at term [Beitins et al., 1973; Dancis et al., 1978; Brown et al., 1993; Blasco et al., 1986]. As for the latter two points, the study on human placenta *in-vitro* did include 2 control enzymes (alkaline phosphatase and N-γ-L glutamyl ß-naphthylaminidase), neither of which correlated with birth weight. However, although these enzymes are stable on storage, the problems with 11ß-HSD storage in these samples, makes any interpretation from these samples inappropriate. The data on treatment of pregnant rats with glucocorticoids or 11ß-HSD inhibitors resulting in low birth weight and offspring hypertension (the effects of 11ß-HSD inhibitors being dependent on intact maternal adrenal glands), strongly suggest a cause and effect relationship [Benediktsson et al., 1993; Lindsay et al., 1994b; Lindsay et al., 1994a; Lindsay & Seckl 1994].

Finally, Section 7 (Human Foetal Osteocalcin and Placental 11ß-HSD, page 172) deals with the possibility of an *in-vivo* marker of foetal glucocorticoid exposure. Cord blood osteocalcin (a specific indicator of osteoblast function), which is a sensitive marker of glucocorticoid exposure in adults, was found to significantly correlate with several indices of term placental 11ß-HSD efficiency (for 11ß-HSD Activity Index, $r = 0.58$, $p < 0.02$). This may prove to be an important research tool in the future, possibly predicting not only hypertension, but also bone disease.

The studies presented here have clearly shown that placental 11ß-HSD plays a major role in controlling glucocorticoid access to the foetus, the efficiency of placental glucocorticoid inactivation correlating with foetal growth in the two species studied. Little is known about the ontogeny of the two 11ß-HSD isoforms or their regulation in humans or in the rat. These will be priorities in the months and years ahead, aided by the recent cloning of human placental 11ß-HSD-2 in our laboratory. Further important avenues to explore will be:

1. The precise mechanism of *in-utero* programming of hypertension by glucocorticoids, including the relevant developmental window (see Section 1.1.3 for possible mechanisms).

2. In addition to the acute effects of nicotine and ethanol on human placental 11ß-HSD presented here, further investigations are warranted on whether the numerous stimuli known to affect foetal growth (some of which cause disproportionate placental enlargement), affect placental 11ß-HSD activity. Some examples are ethanol, smoking, anaemia, malnutrition and diabetes. Indeed, recent evidence indicates a role for placental 11ß-HSD in mediating the effects of protein malnutrition during pregnancy, which in rats causes offspring hypertension [Phillips et al., 1994].

3. The relative contribution of glucocorticoid receptor expression and glucocorticoid levels in the foetus. This can be achieved with cross-breeding experiments of recently available transgenic mice bearing antisense glucocorticoid receptor mRNA constructs [Pepin & Barden 1991].

9. BIBLIOGRAPHY

Abramovich DR, Dacke CG, Elcock C & Page KR (1987). Calcium transport across the isolated dually perfused human placental lobule. *J. Physiol.* 382: 397-410.

Adashi EY, Jones PBC & Hsueh AJW (1981). Synergistic Effects of Glucocorticoids on the Stimulation of Progesterone Production by Follicle-Stimulating Hormone in Cultured Rat Granulosa Cells. *Endocrinology* 109: 1888-1894.

Addison RS, Maguire DJ, Mortimer RH & Cannell GR (1991). Metabolism of prednisolone by the isolated perfused human placental lobule. *J. Steroid Biochem. Molec. Biol.* 39: 83-90.

Addison RS, Maguire DJ, Mortimer RH, Roberts MS & Cannell GR (1993). Pathway and kinetics of prednisolone metabolism in the human placenta. *J. Steroid Biochem. Molec. Biol.* 44: 315-320.

Agarwal AK, Monder C, Eckstein B & White PC (1989). Cloning and expression of rat cDNA encoding corticosteroid 11 beta-dehydrogenase. *J. Biol. Chem.* 264: 18939-18943.

Agarwal AK, Tusie-Luna MT, Monder C & White PC (1990). Expression of 11 beta-hydroxysteroid dehydrogenase using recombinant vaccinia virus. *Mol. Endocrinol.* 4: 1827-1832.

Al-Dujaili EAS & Edwards CRW (1987). The development and application of a direct radioimmunoassay for plasma aldosterone using ^{125}I-labeled ligand - Comparison of three methods. *J. Clin. Endocrinol. Metab.* 46: 105-113.

Albiston AL, Lock P, Burger HG & Krozowski ZS (1990). Cloning and Characterization of the Rat α-Inhibin Gene. *Mol. Cell. Endocrinol.* 68: 121-128.

Albiston AL, Obeyesekere VR, Smith RE & Krozowski ZS (1994). Cloning and tissue distribution of the human 11beta -hydroxysteroid dehydrogenase type 2 enzyme. *Mol. Cell. Endocrinol.* 105: R11-R17.

Alexander G (1978). Factors regulating the growth of the placenta: with comments on the relationship between placental weight and fetal weight. *Abnormal Fetal Growth: Biological Bases and Consequences* (ed Naftolin F), 149-164. Abakon Verlagsgesellschaft, Berlin.

Amelung D, Hübener HJ & Roka J (1953a). Enzymatische umwandlungen von steroiden III uber die einfuhrung der 11-oxygruppe und die reduction der 11-ketogruppe. *Hoppe-Seyler's Z. Physiol. Chem.* 294: 36-48.

Amelung D, Hübener HJ, Roka L & Meyerheim G (1953b). Conversion of cortisone to compound F. *J. Clin. Endocrinol. Metab.* 13: 1125.

Anonymous (1942). People's league of health. *Lancet* 243: 10.

Antoni FA (1986). Hypothalamic control of adrenocorticotropin secretion: advances since the discovery of 41-residue corticotropin-releasing factor. *Endocr. Rev.* 7: 351-378.

Antoni FA (1993). Vasopressinergic control of pituitary adrenocorticotropin secretion comes of age. *Frontiers Neuroendocrinol.* 14: 76-122.

Arai Y & Gorski RA (1968). Critical exposure time for androgenization of the developing hypothalamus in the female rat. *Endocrinology* 82: 1010-1014.

Armanini D, Karbowiak I & Funder JW (1983). Affinity of liquorice derivatives for mineralocorticoid and glucocorticoid receptors. *Clin. Endocrinol.* 19: 609-612.

Arriza JL, Weinberger C & Cerelli G (1987). Cloning of human mineralocorticoid receptor complementary DNA; structural and functional kinship with the glucocorticoid receptor. *Science* 237: 268-275.

Baggia S, Albrecht ED, Babischkin JS & Pepe GJ (1990a). Interconversion of cortisol and cortisone in baboon trophoblast and decidua cells in culture. *Endocrinology* 127: 1735-1741.

Baggia S, Albrecht ED & Pepe GJ (1990b). Regulation of 11 beta-hydroxysteroid dehydrogenase activity in the baboon placenta by estrogen. *Endocrinology* 126: 2742-2748.

Baillie AH, Ferguson MM, Calman KC & Hart DM (1965). Histochemical demonstration of 11-beta-hydroxysteroid dehydrogenase. *J. Endocrinol.* 33: 119-125.

Baldwin DM & Sawyer CH (1974). Effects of Dexamethasone on LH Release and Ovulation in the Cyclic Rat. *Endocrinology* 94: 1397-1403.

Ballard PL (1979). Glucocorticoids and differentiation. (eds Gross F, Grumbach MM, Labhart A, Lipsett MB, Mann T, Samuels LT & Zander J) 493-515. New York, Springer Verlag.

Bambino TH & Hsueh AJW (1981). Direct inhibitory effects of glucocorticoids upon testicular luteinizing hormone receptor and steroidogenesis in vivo and in vitro. *Endocrinology* 108: 2142.

Barker DJP (1988). Childhood causes of adult diseases. *Arch. Dis. Child.* 63: 867-869.

Barker DJP, Osmond C, Golding J, Kuh D & Wadsworth MEJ (1989a). Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease. *Brit. Med. J.* 298: 564-567.

Barker DJP, Osmond C, Winter PD & Margetts B (1989b). Weight in infancy and death from ischaemic heart disease. *Lancet* ii: 577-580.

Barker DJP, Bull AR, Osmond C & Simmonds SJ (1990). Fetal and placental size and risk of hypertension in adult life. *Brit. Med. J.* 301: 259-262.

Barker DJP et al. (1992a). Fetal and infant origins of adult disease. (ed Barker DJP), London, British Medical Journal.

Barker DJP (1992b). The maternal and fetal origins of cardiovascular disease. *Cardiovascular Risk Factors* 2: 4-11.

Barker DJP, Godfrey KM, Osmond C & Bull A (1992a). The relation of fetal length, ponderal index and head circumference to blood pressure and the risk of hypertension in adult life. *Pediatr. Perinatal Epidemiol.* 6: 35-44.

Barker DJP, Meade TW, Fall CHD, Lee A, Osmond C, Stirling Y & Phipps K (1992b). Relation of fetal and infant growth to plasma fibrinogen and factor VII concentrations in adult life. *Brit. Med. J.* 304: 148-152.

Barker DJP, Gluckman PD, Godfrey KM, Harding JE, Owens JA & Robinson JS (1993a). Fetal nutrition and cardiovascular disease in adult life. *Lancet* 341: 938-941.

Barker DJP, Hales CN, Fall CHD, Osmond C, Phipps K & Clark PMS (1993b). Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidaemia (syndrome X): Relation to reduced fetal growth. *Diabetologia* 36: 62-67.

Barker DJP & Osmond C (1986). Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. *Lancet* i: 1077-1081.

Barker DJP & Osmond C (1987). Death rates from stroke in England and Wales predicted from past maternal mortality. *Brit. Med. J.* 295: 83-86.

Beitins IZ, Bayard F, Ances IG, Kowarski A & Migeon CJ (1973). The metabolic clearance rate, blood production, interconversion and transplacental passage of cortisol and cortisone in pregnancy near term. *Pediatr. Res.* 7: 509-519.

Ben-Shlomo Y & Smith GD (1991). Deprivation in infancy or adult life: which is more important for mortality risk? *Lancet* 337: 530-534.

Benediktsson R, Yau JLW, Low S, Brett LP, Cooke BE, Edwards CRW & Seckl JR (1992). 11 beta-Hydroxysteroid dehydrogenase in the rat ovary: high expression in the oocyte. *J. Endocrinol.* 135: 53-58.

Benediktsson R, Lindsay RS, Noble J, Seckl JR & Edwards CRW (1993). Glucocorticoid exposure in utero: new model for adult hypertension. *Lancet* 341: 339-341.

Bian XP, Seidler FJ & Slotkin TA (1992). Promotional role for glucocorticoids in the development of intracellular signalling: enhanced cardiac and renal adenylate cyclase reactivity to ß-adrenergic and non-adrenergic stimuli after low dose fetal dexamethasone exposure. *J. Dev. Physiol.* 17: 289-297.

Bian XP, Seidler FJ & Slotkin TA (1993). Fetal dexamethasone exposure interferes with establishment of cardiac noradrenergic innervation and sympathetic activity. *Teratology* 47: 109-117.

Blanford AT & Murphy BEP (1977). In vitro metabolism of prednisolone, dexamethasone, betamethasone, and cortisol by the human placenta. *Am. J. Obstet. Gynecol.* 127: 264-267.

Blasco MJ, López Bernal A & Turnbull AC (1986). 11 beta-Hydroxysteroid dehydrogenase activity of the human placenta during pregnancy. *Horm. Metab. Res.* 18: 638-641.

Bloxam DL (1985). Human placental energy metabolism: its relevance to in vitro perfusion. *Contr. Gynec. Obstet.* 13: 59-69.

Bonvalet JP, Doignon I, Blot-Chabaud M, Pradelles P & Farman N (1990). Distribution of 11 beta-hydroxysteroid dehydrogenase along the rabbit nephron. *J. Clin. Invest.* 86: 832-837.

Borst JGG, Ten Holt SP & Devries LA (1953). Synergistic action of liquorice and cortisone in Addison's and Simmond's disease. *Lancet* i: 657-663.

Boyd JD & Hamilton WJ (1970). The human placenta. Cambridge, W. Heffer & Sons.

Bradford M (1976). A rapid and sensitive method of quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem.* 72: 248-254.

Brandes JM, Tavoloni N, Potter BJ, Sarkozi L, Shepard MD & Berk PD (1983). A new recycling technique for human placental cotyledon perfusion: application to

studies of the fetomaternal transfer of glucose, inulin, and antipyrine. *Am. J. Obstet. Gynecol.* 146: 800-806.

Brar HS & Rutherford SE (1988). Classification of intrauterine growth retardation. *Seminars in Perinatology* 12: 2-10.

Brem AS, Matheson KL, Latif S & Morris DJ (1993). Activity of 11beta - hydroxysteroid dehydrogenase in toad bladder: Effects of 11-dehydrocorticosterone. *American Journal of Physiology - Renal Fluid and Electrolyte Physiology* 264: F854-F858.

Brem AS, Bina B, Matheson KL, Barnes JL & Morris DJ (1994). Developmental changes in rat renal 11beta -hydroxysteroid dehydrogenase. *Kidney Int.* 45: 679-683.

Brown RW, Chapman KE, Edwards CRW & Seckl JR (1993). Human placental 11ß-hydroxysteroid dehydrogenase: evidence for and partial purification of a distinct NAD-dependent isoform. *Endocrinology* 132: 2614-2621.

Brown RW, Chapman KE, Edwards CRW & Seckl JR (1994). Isolation of 11beta-hydroxysteroid dehydrogenase type 2. *J. Endocrinol.* 140(Suppl): OC34(Abstract).

Buck C & Simpson H (1982). Infant diarrhoea and subsequent mortality from heart disease and cancer. *J. Epidemiol. Commun. Health* 36: 27-30.

Burton AF & Turnell RW (1968). 11-dehydrocorticosteroids in tissues of mice. *Can. J. Biochem.* 46: 497-502.

Bush IE, Hunter SA & Meigs RA (1968). Metabolism of 11-oxygenated steroids. Metabolism in vitro by preparations of liver. *Biochem. J.* 107: 239-258.

Butler N & Alberman ED (1969). Second report of the 1958 British perinatal mortality survey. Edinburgh, Churchill Livingstone.

Campbell AL & Murphy BEP (1977). The maternal-fetal cortisol gradient during pregnancy and at delivery. *J. Clin. Endocrinol. Metab.* 45: 435-440.

Campbell JC, Walker BR, Benediktsson R, Williams BC & Edwards CRW (1992). 11ß-dehydrogenase in rat tissues: organ specific utilisation of NAD or NADP. *J. Endocrinol.* 132(Suppl): 101(Abstract).

Canavan JP & Goldspink DF (1988). Maternal Diabetes in Rats II. Effects on fetal growth and protein turnover. *Diabetes* 37: 1671-1677.

Cannell GR, Galligan JP, Mortimer RH & Thomas MJ (1982). Extraction of steroids from plasma with reversed phase C_{18} cartridges. *Clin. Chim. Acta* 122: 419-422.

Cannell GR, Kluck RM, Hamilton SE, Mortimer RH, Hooper WD & Dickinson RG (1988). Markers of physical integrity and metabolic viability of the perfused human placental lobule. *Clin. Exp. Pharmacol. Physiol.* 15: 837-844.

Card WI, Mitchell W, Strong JA & Taylor NRW (1953). Effects of liquorice and its derivatives on salt and water metabolism. *Lancet* i: 663-668.

Carr BR, Parker CR, Jr., Madden JD & et al. (1981). Maternal plasma adrenocorticotropin and cortisol relationships throughout human pregnancy. *Am. J. Obstet. Gynecol.* 139: 416-422.

Cater J & Gill M (1984). The follow up study: Medical aspects. *Low Birth Weight: A Medical, Psychological and Social Study* (eds Illsley R & Mitchell RG) 1st ed: 191-205. John Wiley and Sons, New York.

Challier JC (1985). Criteria for evaluating perfusion experiments and presentation of results. *Contr. Gynec. Obstet.* 13: 32-39.

Chard T (1987). An introduction to radioimmunoassay and related techniques. (eds Burdon RH & Knippenberg PH) 3rd ed: 1-274. Amsterdam, Elsevier.

Cheng SL, Yang JW, Rifas L, Zhang SF & Avioli LV (1994). Differentiation of human bone marrow osteogenic stromal cells in vitro: Induction of the osteoblast phenotype by dexamethasone. *Endocrinology* 134: 277-286.

Chiswick ML (1985). Intrauterine growth retardation. *Brit. Med. J.* 291: 845-848.

Chomczynski P & Sacchi N (1987). Single-step method of RNA isolation by guanidinum thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162: 156-159.

Contractor SF & Stannard PJ (1983). The Use of AIB Transport to Assess the Suitability of a System of Human Placental Perfusion for Drug Transport Studies. *Placenta* 4: 19-30.

Csaba G, Dobozy O, Lazáry G & Kaizer G (1979). Possibility of long-lasting amplification of insulin receptors by a single treatment at newborn age. *Acta Physiologica Academiae Scientiarum Hungaricae* 534: 478-492.

Csaba G (1980). Phylogeny and ontogeny of hormone receptors: the selection theory of receptor formation and hormonal imprinting. *Biol. Rev.* 55: 47-63.

Csaba G, Rónai A, László V, Darvas Z & Berzétei I (1980). Amplification of hormone Receptors by neonatal Oxytocin and vasopressin treatment. *Horm. Metab. Res.* 12: 28-31.

Dancis J, Jansen V, Levitz M & Rosner W (1978). Effect of protein binding on transfer and metabolism of cortisol in perfused human placenta. *J. Clin. Endocrinol. Metab.* 46: 863-868.

Dancis J, Jansen V & Levitz M (1980). Placental transfer of steroids: effect of binding to serum albumin and to placenta. *Am. J. Physiol.* 238: E208-E213.

Danon A & Prajgrod G (1991). Glucocorticoids regulate prostacyclin synthesis and response to lipopolysaccharide in the rat aorta. *Agents and Actions* 24: 416-423.

Davies J & Glasser SR (1968). Histological and fine ultrastructural observations on the placenta of the rat. *Acta Anat.* 69: 542-608.

Delmas PD, Glorieux FH, Delvin EE et al. (1987). Perinatal serum bone Gla-protein and vitamin D metabolites in preterm and fullterm neonates. *J. Clin. Endocrinol. Metab.* 65: 588-591.

Delmas PD (1990). Biochemical Markers of Bone Turnover for the Clinical Assessment of Metabolic Bone Disease. *Endocrin. Metab. Clin. NA.* 19(1): 1-18.

DemeyPonsart E, Foidart JM, Sulon J & Sodoyez JC (1982). Serum CBG, free and total cortisol and circadian patterns of adrenal function in normal pregnancy. *J. Steroid Biochem.* 16: 165-169.

Dodds H, Maguire DJ, Addison RS, Johnson L, Mortimer RH & Cannell GR (1993). Metabolism of cortisol in the perfused human placental lobule. *Placenta* 14: A17(Abstract).

Dormer RA & France JT (1973). Cortisol and cortisone levels in umbilical cord plasma and maternal plasma of normal pregnancies. *Steroids* 21: 497-510.

Dunn JF, Nisula BC & Rodbard D (1981). Transport of steroid hormones: binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteroid-binding globulin in human plasma. *J. Clin. Endocrinol. Metab.* 53: 58-68.

Duperrex H, Kenouch S, Gaeggeler HP, Seckl JR, Edwards CRW, Farman N & Rossier BC (1993). Rat liver 11beta -hydroxysteroid dehydrogenase complementary deoxyribonucleic acid encodes oxoreductase activity in a mineralocorticoid-responsive toad bladder cell line. *Endocrinology* 132: 612-619.

Eckles CH (1919). *Res. Bull. Min. of Agriculture Exp. Station* 35:

Edwards CRW, Stewart PM, Burt D, Brett LP, McIntyre MA, Sutanto WS, de Kloet ER & Monder C (1988). Localisation of 11ß-hydroxysteroid dehydrogenase-tissue specific protector of the mineralocorticoid receptor. *Lancet* 2: 986-989.

Edwards CRW, Benediktsson R, Lindsay RS & Seckl JR (1993a). Dysfunction of placental glucocorticoid barrier: link between fetal environment and adult hypertension? *Lancet* 341: 355-357.

Edwards CRW, Walker BR, Benediktsson R & Seckl JR (1993b). Congenital and acquired syndromes of apparent mineralocorticoid excess. *J. Steroid Biochem. Molec. Biol.* 45: 1-5.

Eipper BA & Mains RE (1980). Structure and biosynthesis of pro-adrenocorticotropin/endorphin and related peptides. *Endocr. Rev.* 1: 1-27.

Elliot EM & Sapolsky RM (1993). Corticosterone impairs hippocampal neuronal calcium regulation -possible mediating mechanisms. *Brain Research* 602: 84-90.

Epstein MF, Farrell PM, Sparks JW, Pepe GJ, Driscoll SG & Chez RA (1977). Maternal betamethasone and fetal growth and development in the monkey. *Am. J. Obstet. Gynecol.* 127: 261-263.

Evain D, Morera AM & Saez JM (1976). Glucocorticoid receptors in interstitial cells of the rat testis. *J. Steroid Biochem.* 7: 1135.

Exton EH (1979). Regulation of gluconeogenesis by glucocorticoids. *Glucocorticoid Hormone Action* (eds Baxter JD & Rousseau GG) 535-546. Springer Verlag, New York.

Fain JH (1979). Inhibition of glucose transport in fat cells and activation of lipolysis by glucocorticoids. *Glucocorticoid Hormone Action* (eds Baxter JD & Rousseau GG) 547-560. Springer Verlag, New York.

Fall CHD, Barker DJP, Osmond C, Winter PD, Clark PMS & Hales CN (1992). Relation of infant feeding to adult serum cholesterol concentration and death from ischaemic heart disease. *Brit. Med. J.* 304: 801-805.

Ferguson MM & Christie GA (1967). Distribution of hydroxysteroid dehydrogenases in the placentae and foetal membranes of various mammals. *J. Endocrinol.* 38: 291-306.

Forsdahl A (1977). Are poor living conditions in childhood and adolescence an important risk factor for arteriosclerotic heart disease? *British Journal of Preventive Social Medicine* 31: 91-95.

Fuller PJ & Verity K (1990). Colonic sodium-potassium adenosine triphopsphate subunit gene expression: ontogeny and regulation by adrenocortical steroids. *Endocrinology* 127: 32-38.

Funder JW, Pearce PT, Smith R & Smith AI (1988). Mineralocorticoid action: target tissue specificity is enzyme, not receptor, mediated. *Science* 242: 583-585.

Gaeggeler HP, Edwards CRW & Rossier BC (1989). Steroid metabolism determines mineralocorticoid specificity in the toad bladder. *Am. J. Physiol.* 257: F690-F695.

Gennser G, Rymark P & Isberg PE (1988). Low birth weight and risk of high blood pressure in adulthood. *Brit. Med. J.* 296: 1498-1500.

Ghraf R, Vetter U, Zandveld JM & Schriefers H (1975). Organ-Specific Ontogenesis of Steroid Hormone Metabolizing enzyme Activities in the Rat. *Acta Endocrinol.* 79: 192-201.

Giannopoulos G, Jackson K & Tulchinsky D (1982). Glucocorticoid metabolism in human placenta, decidua, myometrium and fetal membranes. *J. Steroid Biochem.* 17: 371-374.

Gill GN (1972). Progress in endocrinology and metabolism: mechanism of ACTH action. *Metabolism* 21: 571-588.

Godfrey KM, Redman CWG, Barker DJP & Osmond C (1991). The effect of maternal anaemia and iron deficiency on the ratio of fetal weight to placental weight. *Brit. J. Obstet. Gynaec.* 98: 886-891.

Goland RS, Jozak S, Warren WB, Conwell IM, Stark RI & Tropper PJ (1993). Elevated levels of umbilical cord plasma corticotropin-releasing hormone in growth retarded fetuses. *J. Clin. Endocrinol. Metab.* 77: 1174-1179.

Grollman A & Grollman EF (1962). The teratogenic induction of hypertension. *J. Clin. Invest.* 41: 710-714.

Gunberg DL (1957). Some effects of exogenous hydrocortisone on pregnancy in the rat. *Anat. Rec.* 129: 133-153.

Gustafsson GA & Stenberg A (1974a). Neonatal programming of androgen responsiveness of liver of adult rats. *J. Biol. Chem.* 249: 719-723.

Gustafsson GA & Stenberg A (1974b). Irreversible androgenic programming at birth of microsomal and soluble rat liver enzymes active on 4-androstene-3,17-dione and 5-alpha, androstane-3-alpha,17-beta-diol. *J. Biol. Chem.* 249: 711-718.

Haider SG, Passia D & Rommert FF (1990). Histochemical demonstration of 11 beta-hydroxysteroid dehydrogenase as a marker for Leydig cell maturation in rat. *Acta Histochem.* 38(Suppl): 203-207.

Hales CN, Barker DJP, Clark PMS, Cox LJ, Fall C, Osmond C & Winter PD (1991). Fetal and infant growth and impaired glucose tolerance at age 64. *Brit. Med. J.* 303: 1019-1022.

Hamilton BP, Zadik Z, Edwin CM, Hamilton JH & Kowarski AA (1979). Effect of adrenal suppression with dexamethasone in essential hypertension. *J. Clin. Endocrinol. Metab.* 48: 848-853.

Hammond J (1944). Physiological factors affecting birth weight. *P. Nutr. Soc.* 2: 8-12.

Hart DM (1966). The histochemical distribution of hydroxysteroid dehydrogenases in the human foetal membranes at term. *Histochemie* 6: 17-23.

Hauguel S, Challier JC, Cedard L & Olive G (1983). Metabolism of the human placenta perfused in vitro: glucose transfer and utilization, O_2 consumption, lactate and ammonia production. *Pediatr. Res.* 17: 729-732.

Hechter O, Zaffaroni A, Jacobsen RP, Levy H, Jeanloz RW, Schenker V & Pincus G (1951). The nature and biogenesis of the adrenal secretory product. *Recent Prog. Horm. Res.* 6: 215-241.

Heller CL, Weisenberg LS, Ortí E & de Nicola AF (1988). Steps of glucocorticoid action in normal and diabetic rat placenta. *J. Steroid Biochem.* 31: 119-123.

Hoefnagels WHL & Kloppenborg PW (1983). Antimineralocorticoid effect of dexamethasone in subjects treated with glycyrrhetinic acid. *J. Hypertens.* 1(Suppl 2): 313-315.

Homma M, Oka K, Niitsuma T & Itoh H (1994). A novel 11ß-hydroxysteroid inhibitor contained in Saiboku-To, a herbal remedy for steroid dependent bronchial asthma. *J. Pharm. Pharmacol.* 46: 305-309.

Hsueh AJW & Erickson GF (1978). Glucocorticoid Inhibition of FSH-Induced Estrogen Production in Cultured Rat Granulosa Cells. *Steroids* 32: 639-648.

Huggett A (1944). Diet in pregnancy. *P. Nutr. Soc.* 2: 20-27.

Hundertmark S, Ragosch V, Schein B, Buhler H, Lorenz U, Fromm M & Weitzel HK (1994). Gestational age dependence of 11beta -hydroxysteroid dehydrogenase and its relationship to the enzymes of phosphatidylcholine synthesis in lung and liver of fetal rat. *Biochimica et Biophysica Acta - Lipids and Lipid Metabolism* 1210: 348-354.

Hübener HJ, Fukushima DK & Gallagher TF (1956). Substrate specificity of enzymes reducing the 11- and 20-keto groups of steroids. *J. Biol. Chem.* 220: 499-511.

Illsley NP, Aarnoudse JG, Penfold P, Bardsley SE, Coade SB, Stacey TE & Hytten FE (1984). Mechanical and metabolic viability of a placental perfusion system in vitro under oxygenated and anoxic conditions. *Placenta* 5: 213-225.

Inazu N, Iwata N & Satoh T (1990). Inhibitory effect of glucocorticoid and stimulatory effect of human chorionic gonadotropin on ovarian carbonyl reductase in rats. *Life Sci.* 46: 841-848.

Jauniaux E, Moscoso JG, Vanesse M, Campbell S & Driver M (1991). Perfusion fixation for placental morphologic investigation. *Hum. Pathol.* 22: 442-449.

Jeffcoate WJ, Silverstone JT, Edwards CRW & et al (1979). Psychiatric manifestations of Cushing's syndrome: response to lowering of plasma cortisol. *Q. J. Med.* 48: 465-472.

Jia XC & Hsueh AJW (1990). Synergistic Effect of Glucocorticoids and Androgens on The Hormonal Induction of Tissue Plasminogen Activator Activity and Messenger Ribonucleic Acid Level in Granulosa Cells. *Mol. Cell. Endocrinol.* 68: 143-151.

John ME, John MC, Boggaram V, Simpson ER & Waterman MR (1986). Transcriptional regulation of steroid hydroxylase genes by corticotropin. *P. Natl. Acad. Sci. USA.* 83: 4715-4719.

Johnson JWC, Mitzner W, Beck JC, London WT, Sly DL, Lee PA, Khouzami VA & Cavalieri RL (1981). Long-term effects of betamethasone on fetal development. *Am. J. Obstet. Gynecol.* 141: 1053-1064.

Jónsson G (1830). Safn af íslenzkum orðskviðum, fornmælum, heilræðum, snilliyrðum, sannmælum og málsgreinum, samanlesið og í stafrófsröð sett af Guðmundi Jónssyni prófasti í Snæfellsnessýslu og presti í Staðarstaðarsókn. 1-424. Hið íslenzka bókmenntafélag, Copenhagen.

Kageyama Y, Suzuki H & Saruta T (1992). Glycyrrhizin induces mineralocorticoid activity through alterations in cortisol metabolism in the human kidney. *J. Endocrinol.* 135: 147-152.

Katz VL, Thorp JM, Jr. & Bowes WA, Jr. (1990). Severe symmetric intrauterine growth retardation associated with the topical use of triamcinolone. *Am. J. Obstet. Gynecol.* 162: 396-397.

Kaufmann P (1985). Influence of ischemia and artificial perfusion on placental ultrastructure and morphometry. *Contr. Gynec. Obstet.* 13: 18-26.

Kirkinen P, Jouppila P, Korwula A, Vuori J & Pukka M (1983). The effect of caffeine on placental and fetal blood flow in human pregnancy. *Am. J. Obstet. Gynecol.* 147: 939-942.

Kornel L (1994). Colocalisation of 11ß-hydroxysteroid dehydrogenase and mineralocorticoid receptors in cultured vascular smooth muscle cells. *Am. J. Hypertens.* 7: 100-103.

Kramer MS, Olivier M, McLean FH, Willis DM & Usher RH (1990). Impact of intrauterine growth retardation and body proportionality on fetal and neonatal outcome. *Pediatrics* 86: 707-713.

Kreuz LE, Rose RM & Jennings JR (1972). Suppression of plasma testosterone levels and psychological stress. *Arch. Gen. Psychiatry* 26: 479.

Krieger DT, Allen W, Rizzo F & et al (1971). Characterization of the normal temporal pattern of plasma corticosteroid levels. *J. Clin. Endocrinol. Metab.* 32: 266-284.

Krozowski ZS, Stuchbery S, White PC, Monder C & Funder JW (1990). Characterization of 11 beta-hydroxysteroid dehydrogenase gene expression:

identification of multiple unique forms of messenger ribonucleic acid in the rat kidney. *Endocrinology* 127: 3009-3013.

Krozowski ZS, Obeyesekere V, Smith R & Mercer WR (1992). Tissue-specific expression of an 11 beta-hydroxysteroid dehydrogenase with a truncated N-terminal domain. A potential mechanism for differential intracellular localization within mineralocorticoid target cells. *J. Biol. Chem.* 267: 2569-2574.

Krozowski ZS (1994). Cloning of the type 2 isoform of 11ß-OHSD. *Endocr. Res.* (Abstract In Press).

Krozowski ZS & Funder JW (1983). Renal mineralocorticoid receptors and hippocampal corticosterone-binding species have identical intrinsic steroid specificity. *P. Natl. Acad. Sci. USA.* 80: 6056-6060.

Krüger H & Arias-Stella J (1970). The placenta and the newborn infant at high altitudes. *Am. J. Obstet. Gynecol.* 106: 586-591.

Kuhn DC, Crawford MA, Gordon GB & Stuart MJ (1988). Aspects of in vitro placental perfusion: effects of hyperoxia and phenol red. *Placenta* 9: 201-213.

Laake H (1960). The action of corticosteroids on the renal absorption of calcium. *Acta Endocrinol.* 34: 60-64.

Lakshmi V, Sakai RR, McEwen BS & Monder C (1991). Regional distribution of 11 beta-hydroxysteroid dehydrogenase in rat brain. *Endocrinology* 128: 1741-1748.

Lakshmi V, Nath N & Muneyyirci-Delale O (1993). Characterisation of 11ß-hydroxysteroid dehydrogenase of human placenta: evidence for the existence of two species of 11ß-hydroxysteroid dehydrogenase. *J. Steroid Biochem. Molec. Biol.* 45: 391-397.

Lakshmi V & Monder C (1985). Evidence for independent 11-oxidase and 11-reductase activities of 11 beta-hydroxysteroid dehydrogenase: enzyme latency, phase transitions, and lipid requirements. *Endocrinology* 116: 552-560.

Langley SC & Jackson AA (1994). Increased systolic blood pressure in adult rats induced by fetal exposure to maternal low protein diets. *Clin. Sci.* 86: 217-222.

Langman J (1981). Fetal period. *Medical Embryology* 4th ed: 74-82. Williams & Wilkins, Baltimore.

LaRocque S, O'Donnell D, Gianoulakis C, Seckl JR & Meaney MJ (1992). Postnatal handling in the rat alters hippocampal glucocorticoid receptor gene expression. *Society for Neuroscience* A204.7(Abstract).

Latif SA, Hartman LR, Souness GW & Morris DJ (1994). Possible endogenous regulators of steroid inactivating enzymes and glucocorticoid-induced Na+ retention. *Steroids* 59: 352-356.

Law CM, Barker DJP, Bull AR & Osmond C (1991). Maternal and fetal influences on blood pressure. *Arch. Dis. Child.* 66: 1291-1295.

Law CM, Barker DJP, Osmond C, Fall CHD & Simmonds SJ (1992). Early growth and abdominal fatness in adult life. *J. Epidemiol. Commun. Health* 46: 184-186.

Law CM, De Swiet M, Osmond C, Fayers PM, Barker DJP, Cruddas AM & Fall CHD (1993). Initiation of hypertension in utero and its amplification throughout life. *Brit. Med. J.* 306: 24-27.

Leong MK & Murphy BEP (1976). Cortisol levels in maternal venous and umbilical cord arterial and venous serum at vaginal delivery. *Am. J. Obstet. Gynecol.* 124: 471-473.

Lever AF & Harrap SB (1992). Essential hypertension: a disorder of growth with origins in childhood? *J. Hypertens.* 10: 101-120.

Levitz M, Jansen V & Dancis J (1978). The transfer and metabolism of corticosteroids in the perfused human placenta. *Am. J. Obstet. Gynecol.* 132: 363-366.

Li J, Saunders JC, Gilmour RS, Silver M & Fowden AL (1993). Insulin-like growth factor-II messenger ribonucleic acid expression in fetal tissues of the sheep during late gestation: Effects of cortisol. *Endocrinology* 132: 2083-2089.

Lindsay RS, Noble J, Edwards CRW & Seckl JR (1994a). 11ß-hydroxysteroid dehydrogenase inhibition in utero in the rat is associated with insulin resistance in middle aged offspring. *Scot. Med. J.* 39: 59(Abstract).

Lindsay RS, Noble J, Edwards CRW & Seckl JR (1994b). Maternal carbenoxolone treatment reduces birthweight in the rat. *J. Endocrinol.* 140(Suppl): P18(Abstract).

Lindsay RS & Seckl JR (1994). (Unpublished)

Low SC, Assaad SN, Rajan V, Chapman KE, Edwards CRW & Seckl JR (1993). Regulation of 11beta -hydroxysteroid dehydrogenase by sex steroids in vivo: Further evidence for the existence of a second dehydrogenase in rat kidney. *J. Endocrinol.* 139: 27-35.

Low SC, Chapman KE, Edwards CRW & Seckl JR (1994). Liver-type 11ß-hydroxysteroid dehydrogenase cDNA encodes reductase not dehydrogenase activity in intact mammalian COS-7 cells. *J. Mol. Endocrinol.* 13: 167-174.

López Bernal A, Anderson ABM, Parry DM & Turnbull AC (1980a). Evidence that fetal membranes are not involved in cortisol metabolism: study of dichorionic twin pregnancies. *Am. J. Obstet. Gynecol.* 138: 1168-1172.

López Bernal A, Flint AP, Anderson ABM & Turnbull AC (1980b). 11ß-Hydroxysteroid dehydrogenase activity (E.C. 1.1.1.146) in human placenta and decidua. *J. Steroid Biochem.* 13: 1081-1087.

López Bernal A, Anderson ABM & Turnbull AC (1982a). Cortisol:cortisone interconversion by human decidua in relation to parturition: effect of tissue manipulation on 11 beta-hydroxysteroid dehydrogenase activity. *J. Endocrinol.* 93: 141-149.

López Bernal A, Anderson ABM & Turnbull AC (1982b). The lack of influence of labor on human placental 11ß-hydroxysteroid dehydrogenase activity. *J. Clin. Endocrinol. Metab.* 54: 1251-1254.

López Bernal A & Craft IL (1981). Corticosteroid metabolism in vitro by human placenta, fetal membranes and decidua in early and late gestation. *Placenta* 2: 279-285.

Lukert BP & Adams JS (1976). Calcium and phosphorus homeostasis in man: effects of corticosteroids. *Arch. Intern. Med.* 136: 1249-1253.

Lundbland JR & Roberts JL (1988). Regulation of the proopiomelanocortin gene expression in the pituitary. *Endocr. Rev.* 9: 135-158.

Luo J, Reid RE & Murphy LM (1990). Dexamethasone increases hepatic insulin-like growth factor binding protein-1 (IGFBP-1) mRNA and serum IGFBP-1 concentrations in the rat. *Endocrinology* 127: 1456-1462.

MacKenzie MA, Hoefnagels WH, Jansen RW, Benraad TJ & Kloppenborg PW (1990). The influence of glycyrrhetinic acid on plasma cortisol and cortisone in healthy young volunteers. *J. Clin. Endocrinol. Metab.* 70: 1637-1643.

Malbon CC & Hadcock JR (1988). Evidence that glucocorticoid response elements in the 5'-noncoding region of the hamster beta 2-adrenergic receptor gene are obligate for glucocorticoid regulation of receptor mRNA levels. *Biochem. Bioph. Res. Co.* 1540: 6760-6681.

Mantero F, Tedde R, Opocher G, Fulgheri PD, Arnaldi G & Ulick S (1994). Apparent mineralocorticoid excess type II. *Steroids* 59: 80-83.

Martín A, Palacín M, Lasunción MA & Herrera E (1990). Fetal/maternal plasma amino acid relationships in the streptozotocin diabetic rat. *Endocrine and Biochemical Development of the Fetus and Neonate* (eds Cuezva JM, Pascual-Leone AM & Patel MS) 277-282. New York, Plenum Press.

Maser E, Friebertshauser J & Mangoura SA (1994). Ontogenic pattern of carbonyl reductase activity of 11beta -hydroxysteroid dehydrogenase in mouse liver and kidney. *Xenobiotica* 24: 109-117.

Maser E & Bannenberg G (1994). The purification of 11beta -hydroxysteroid dehydrogenase from mouse liver microsomes. *J. Steroid Biochem. Molec. Biol.* 48: 257-263.

Mason HL (1950). Isolation of adrenal cortical hormones from urine: 17-hydroxycorticosterone and 17-hydroxy-11-dehydrocorticosterone. *J. Biol. Chem.* 182: 131-149.

Masters PW, Jones RG, Purves DA, Cooper EH & Cooney JM (1994). Commercial assays for serum osteocalcin give clinically discordant results. *Clin. Chem.* 40: 358-363.

Mayer JS (1985). Biochemical effects of corticosteroid on neural tissues. *Physiol. Dev.* 65: 1020.

McConway MG & Chapman RS (1986). Development and evaluation of a simple, direct, solid-phase radioimmunoassay of serum cortisol from readily available reagents. *Clin. Chim. Acta* 158: 59-70.

Meaney MJ, O'Donnell D, Viau V & et al (1993). Corticosteroid receptors in the rat brain and pituitary during development and hypothalamic-pituitary-adrenal function. 163-201.

Meigs RA & Engel LL (1961). The metabolism of adrenocortical steroids by human tissues. *Endocrinology* 69: 152-162.

Mercer WR, Obeyesekere V, Smith R & Krozowski ZS (1993). Characterization of 11ß-HSD1B gene expression and enzymatic activity. *Mol. Cell. Endocrinol.* 92: 247-251.

Mercer WR & Krozowski ZS (1992). Localization of an 11 beta hydroxysteroid dehydrogenase activity to the distal nephron. Evidence for the existence of two species of dehydrogenase in the rat kidney. *Endocrinology* 130: 540-543.

Meulenberg PM & Hofman JA (1990). Differences between concentrations of salivary cortisol and cortisone and of free cortisol and cortisone in plasma during pregnancy and postpartum. *Clin. Chem.* 36: 70-75.

Michael AE, Gregory L, Walker SM, Antoniw JW, Shaw RW, Edwards CRW & Cooke BA (1993a). Ovarian 11beta -hydroxysteroid dehydrogenase: Potential predictor of conception by *in-vitro* fertilisation and embryo transfer. *Lancet* 342: 711-712.

Michael AE, Pester LA, Curtis P, Shaw RW, Edwards CRW & Cooke BA (1993b). Direct inhibition of ovarian steroidogenesis by cortisol and the modulatory role of 11ß-hydroxysteroid dehydrogenase. *Clin. Endocrinol.* 38: 641-644.

Michael AE & Cooke BA (1994). A working hypothesis for the regulation of steroidogenesis and germ cell development in the gonads by glucocorticoids and 11beta -hydroxysteroid dehydrogenase (11beta HSD). *Mol. Cell. Endocrinol.* 100: 55-63.

Miller HC & Hassanein K (1974). Maternal smoking and fetal growth of full-term infants. *Pediatrics* 8: 960.

Miller RK, Wier PJ, Maulik D & di Sant'Agnese PA (1985). Human placenta in vitro: characterization during 12 h of dual perfusion. *Contr. Gynec. Obstet.* 13: 77-84.

Moisan MP, Seckl JR, Brett LP, Monder C, Agarwal AK, White PC & Edwards CRW (1990a). 11Beta-Hydroxysteroid Dehydrogenase Messenger Ribonucleid Acid Expression, Bioactivity and Immunoreactivity in Rat Cerebellum. *J. Neuroendocrinol.* 2: 853-858.

Moisan MP, Seckl JR & Edwards CRW (1990b). 11 beta-hydroxysteroid dehydrogenase bioactivity and messenger RNA expression in rat forebrain: localization in hypothalamus, hippocampus, and cortex. *Endocrinology* 127: 1450-1455.

Moisan MP, Edwards CRW & Seckl JR (1992a). Differential promoter usage by the rat 11 beta-hydroxysteroid dehydrogenase gene. *Mol. Endocrinol.* 6: 1082-1087.

Moisan MP, Edwards CRW & Seckl JR (1992b). Ontogeny of 11 beta-hydroxysteroid dehydrogenase in rat brain and kidney. *Endocrinology* 130: 400-404.

Monder C, Stewart PM, Lakshmi V, Valentino R, Burt D & Edwards CRW (1989). Licorice inhibits corticosteroid 11 beta-dehydrogenase of rat kidney and liver: in vivo and in vitro studies. *Endocrinology* 125: 1046-1053.

Monder C (1991). Heterogeneity of 11 beta-hydroxysteroid dehydrogenase in rat tissues. *J. Steroid Biochem. Molec. Biol.* 40: 533-536.

Monder C, Miroff V, Marandici A & Hardy MP (1994a). 11beta-Hydroxysteroid dehydrogenase alleviates glucocorticoid-mediated inhibition of steroidogenesis in rat Leydig cells. *Endocrinology* 134: 1199-1204.

Monder C, Sakai RR, Miroff Y, Blanchard DC & Blanchard RJ (1994b). Reciprocal changes in plasma corticosterone and testosterone in stressed male rats maintained in a visible burrow system: Evidence for a mediating role of testicular 11beta-hydroxysteroid dehydrogenase. *Endocrinology* 134: 1193-1198.

Monder C & Lakshmi V (1990). Corticosteroid 11 beta-dehydrogenase of rat tissues: immunological studies. *Endocrinology* 126: 2435-2443.

Monder C & White PC (1993). 11ß-hydroxysteroid dehydrogenase. *Vitamins and Hormones* 47: 187-271.

Moore CCD, Mellon SH, Murai J, Siiteri PK & Miller WL (1993). Structure and function of the hepatic form of 11beta -hydroxysteroid dehydrogenase in the squirrel monkey, an animal model of glucocorticoid resistance. *Endocrinology* 133: 368-375.

Mornet E, Dupont J, Vitek A & White PC (1989). Characterization of two genes encoding human steroid 11 beta-hydroxylase (P-450(11) beta). *J. Biol. Chem.* 264: 20961-20967.

Morris DJ, Semafuko WE, Latif SA, Vogel B, Grimes CA & Sheff MF (1992). Detection of glycyrrhetinic acid-like factors (GALFs) in human urine. *Hypertension* 20: 356-360.

Morrison N & Eisman J (1993). Role of the negative glucocorticoid regulatory element in glucocorticoid repression of the human osteocalcin promoter. *J Bone Miner Res* 8: 969-975.

Morton NE (1955). The inheritance of human birth weight. *Am. Hum. Genet.* 20: 125.

Mott GE (1986). Deferred effects of breastfeeding versus formula feeding on serum lipoprotein concentrations and cholesterol metabolism in baboons. *The Breastfed Infant: A Model for Performance. Report of the Ninety-first Ross Conference on Paediatric Research* (eds Filer LJJ & Fomon SJ) 144-149. Ross Laboratories, Columbus, Ohio.

Mountjoy KG, Robbins LS, Mortrud MT & Cone RD (1992). The cloning of a family of genes that encode the melanocortin receptors. *Science* 257: 1248-1251.

Munck A (1962). Studies on the mode of action of glucocorticoids in rats. II. The effects in vivo and in vitro on net glucose uptake by isolated adipose tissue. *Biochim. Biophys. Acta* 57: 318-326.

Murphy BEP (1973). Steroid arteriovenous differences in umbilical cord plasma: evidence of cortisol production by the human fetus in early gestation. *J. Clin. Endocrinol. Metab.* 36: 1037-1038.

Murphy BEP, Clark SJ, Donald IR, Pinsky M & Vedady DL (1974). Conversion of maternal cortisol to cortisone during placental transfer to the human fetus. *Am. J. Obstet. Gynecol.* 118: 538-541.

Murphy BEP (1977a). Chorionic membrane as an extra-adrenal source of foetal cortisol in human amniotic fluid. *Nature* 266: 179-181.

Murphy BEP (1977b). Conversion of cortisol to cortisone by the human uterus and its reversal in pregnancy. *J. Clin. Endocrinol. Metab.* 44: 1214-1217.

Murphy BEP (1979). The influence of serum proteins on the metabolism of cortisol by the human placenta. *J. Steroid Biochem.* 10: 387-392.

Murphy BEP (1981). Ontogeny of cortisol-cortisone interconversion in human tissues: a role for cortisone in human fetal development. *J. Steroid Biochem.* 14: 811-817.

Murphy BEP (1983). Human fetal serum cortisol levels at delivery: a review. *Endocr. Rev.* 4: 150-154.

NarayFejesToth A, Watlington CO & Fejes-Toth G (1991). 11 beta-Hydroxysteroid dehydrogenase activity in the renal target cells of aldosterone. *Endocrinology* 129: 17-21.

NarayFejesToth A, Rusvai E, Denault DL, St Germain DL & Fejes-Toth G (1993). Expression and characterization of a new species of 11beta -hydroxysteroid dehydrogenase in Xenopus oocytes. *Am. J. Physiol. - Renal Fluid and Electrolyte Physiology* 265: F896-F900.

NarayFejesToth A, Rusvai E & Fejes-Toth G (1994a). Mineralocorticoid receptors and 11ß-steroid dehydrogenase activity in renal principal and intercalated cells. *Am. J. Physiol.* 266: F76-F80.

NarayFejesToth A, Rusvai E & Fejes-Toth G (1994b). Is the renal type III corticosteroid-binding site the collecting duct- specific isoform of 11beta -hydroxysteroid dehydrogenase? *Endocrinology* 134: 1671-1675.

NarayFejesToth A & Fejes-Toth G (1994). 11beta -Hydroxysteroid dehydrogenase in renal collecting duct cells. *Steroids* 59: 105-110.

Neher R (1964). Steroid chromatography. 122. London, Elsevier.

Nelson DR, Kamataki T, Waxman DJ, Guengerich FP, Estabrook RW, Feyereisen R, Gonzales FJ, Coon MJ, Gunsalus IC, Gotoh O, Okuda N & Nebert DW (1993). The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. *DNA Cell Biol.* 12: 1-51.

Neumann A, Haider SG & Hilscher B (1993). Temporal coincidence of the appearance of elongated spermatids and of histochemical reaction of 11ß-hydroxysteroid dehydrogenase in rat Leydig cells. *Andrologia* 25: 263-269.

New MI, Levine LS, Biglieri EG, Pariera J & Ulick S (1977). Evidence for an unidentified steroid in a child with apparent mineralocorticoid hypertension. *J. Clin. Endocrinol. Metab.* 44: 924-933.

Nikkila H, Tannin GM, New MI, Taylor NF, Kalaitzoglou G, Monder C & White PC (1993). Defects in the HSD11 gene encoding 11ß-hydroxysteroid dehydrogenase are not found in patients with apparent mineralocorticoid excess or 11-oxoreductase deficiency. *J. Clin. Endocrinol. Metab.* 77: 687-691.

Obeid J, Curnow KM, Aisenberg J & White PC (1993). Transcripts originating in intron 1 of the HSD11 (11ß-hydroxysteroid dehydrogenase) gene encode a truncated polypeptide that is enzymatically inactive. *Mol. Endocrinol.* 7: 154-160.

Oberfield SE, Levine LS, Carey RM, Grieg F, Ulick S & New MI (1983). Metabolic and blood pressure responses to hydrocortisone in the syndrome of apparent mineralocorticoid excess. *J. Clin. Endocrinol. Metab.* 56: 332-339.

Oliver MH, Harding JE, Breier BH, Evans PC & Gluckman PD (1993). Glucose but not a mixed amino acid infusion regulates plasma insulin- like growth factor-I concentrations in fetal sheep. *Pediatr. Res.* 34: 62-65.

Ooi GT, Orlowski CC, Brown AL, Becker RE, Unterman TG & Rechler MM (1990). Different tissue distribution and hormonal regulation of messenger RNAs encoding rat insulin-like growth factor-binding proteins-1 and -2. *Mol. Endocrinol.* 4: 321-328.

Orth DN, Kovacs WJ & DeBold CR (1992a). The Adrenal Cortex. *Williams Textbook of Endocrinology* (eds Wilson JD & Foster DW) 8th ed: 489-619. W.B. Saunders company, Philadelphia.

Orth DN, Kovacs WJ & DeBold CR (1992b). The Adrenal Cortex. *Williams Textbook of Endocrinology* (eds Wilson JD & Foster DW) 8th ed: 511-513. W.B. Saunders Company, Philadelphia.

Orth DN, Kovacs WJ & DeBold CR (1992c). The Adrenal Cortex. *Williams Textbook of Endocrinology* (eds Wilson JD & Foster DW) 8th ed: 517-518. W.B. Saunders Company, Philadelphia.

Orth DN, Kovacs WJ & DeBold CR (1992d). The Adrenal Cortex. *Williams Textbook of Endocrinology* (eds Wilson JD & Foster DW) 8th ed: 514. W.B. Saunders Company, Philadelphia.

Orth DN, Kovacs WJ & DeBold CR (1992e). The Adrenal Cortex. *Williams Textbook of Endocrinology* (eds Wilson JD & Foster DW) 8th ed: 509-511. W.B. Saunders Company, Philadelphia.

Orth DN, Kovacs WJ & DeBold CR (1992f). The Adrenal Cortex. *Williams Textbook of Endocrinology* (eds Wilson JD & Foster DW) 8th ed: 519. W.B. Saunders Company, Philadelphia.

Osinski PA (1960). Steroid 11ß-ol dehydrogenase in human placenta. *Nature* 187: 777.

Osmond C, Barker DJP & Slattery JM (1990). Risk of death from cardiovascular disease and chronic bronchitis determined by place of birth in England and Wales. *J. Epidemiol. Commun. Health* 44: 139-141.

Ounsted M & Ounsted C (1966). Maternal regulations of intrauterine growth. *Nature* 187: 777.

Page KR (1993). The physiology of the human placenta. 1st ed: 1-164. London, UCL Press.

Partsch CJ, Sippel WG, MacKenzie IZ & Aynsley-Green A (1991). The stereoid hormonal milieu of the undisturbed human fetus and mother at 16-20 weeks gestation. *J. Clin. Endocrinol. Metab.* 73: 969-974.

Pasqualini JR, Nguyen BL, Uhrich F, Wiqvist N & Diczfalusy E (1970). Cortisol and cortisone metabolism in the human foeto-placental unit at midgestation. *J. Steroid Biochem.* 1: 209-219.

Penfold P, Drury L, Simmonds R & Hytten FE (1981). Studies of a single placental cotyledon in vitro: I. The preparation and its viability. *Placenta* 2: 149-154.

Pepe GJ, Waddell BJ, Stahl SJ & Albrecht ED (1988). The regulation of transplacental cortisol-cortisone metabolism by estrogen in pregnant baboons. *Endocrinology* 122: 78-83.

Pepe GJ & Albrecht ED (1984a). Comparison of cortisol-cortisone interconversion in vitro by the human and baboon placenta. *Steroids* 44: 229-240.

Pepe GJ & Albrecht ED (1984b). Transuteroplacental metabolism of cortisol and cortisone during mid- and late gestation in the baboon. *Endocrinology* 115: 1946-1951.

Pepe GJ & Albrecht ED (1985). Transplacental corticosteroid metabolism during baboon pregnancy. *Research in Perinatal Medicine (IV), Perinatal Endocrinology* (eds Pepe GJ & Albrecht ED) 201. Perinatology Press, New York.

Pepe GJ & Albrecht ED (1987). Fetal regulation of transplacental cortisol-cortisone metabolism in the baboon. *Endocrinology* 120: 2529.

Pepin MC & Barden N (1991). Decreased glucocorticoid receptor activity following glucocorticoid receptor antisense RNA gene fragment transfection. *Molecular and Cellular Biology* 11: 1647-1653.

Phillipou G & Higgins BA (1985). A new defect in the peripheral conversion of cortisone to cortisol. *J. Steroid Biochem.* 22: 435-436.

Phillips DM, Lakshmi V & Monder C (1989). Corticosteroid 11 beta-dehydrogenase in rat testis. *Endocrinology* 125: 209-216.

Phillips GJ, Langley-Evans SC, Benediktsson R, Seckl JR, Edwards CRW & Jackson AA (1994). The role of dietary protein restriction during pregnancy on the activity of placental 11ß-hydroxysteroid dehydrogenase. *P. Nutr. Soc.* (Abstract In Press).

Piercy EC, Michael AE, Pester LA, Shaw RW, Edwards CRW & Cooke BA (1993). *J. Endocrinol.* 139(Suppl): P3(Abstract).

Plotz CM, Knowlton AI & Ragan C (1952). The natural history of Cushing's syndrome. *Am. J. Med.* 13: 597-614.

Price PA & Nishimoto SK (1980). Radioimmunoassay for the vitamin K - dependent protein of bone and its discovery in plasma. *P. Natl. Acad. Sci. USA.* 77: 2234-2238.

Price WA, Stiles AD, MoatsStaats BM & DErcole AJ (1992). Gene expression of insulin-like growth factors (IGFs), the type 1 IGF receptor, and IGF-binding proteins in dexamethasone-induced fetal growth retardation. *Endocrinology* 130: 1424-1432.

Putney DJ, Henson MC, Pepe GJ & Albrecht ED (1991). Influence of the fetus and estrogen on maternal serum growth hormone, insulin-like growth factor-II, and

epidermal growth factor concentrations during baboon pregnancy. *Endocrinology* 129: 3109-3117.

Quinn SJ & Williams GH (1988). Regulation of aldosterone secretion. *Annu. Rev. Physiol.* 50: 409-426.

Ravelli GP, Stein ZA & Susser MV (1976). Obesity in young men after famine exposure in utero and early infancy. *N. Engl. J. Med.* 295: 349-353.

Redei E, Halasz I, Li LF, Prystowsky MB & Aird F (1993). Maternal adrenalectomy alters the immune and endocrine functions of fetal alcohol-exposed male offspring. *Endocrinology* 133: 452-460.

Reich R, Miskin R & Tsafriri A (1985). Follicular Plasminogen Activator: Involvement in ovulation. *Endocrinology* 116: 516-521.

Reinisch JM, Simon NG, Karow WG & Gandelman R (1978). Prenatal Exposure to Prednisone in Humans and Animals Retards Intrauterine Growth. *Science* 202: 436-438.

Riddle MC & McDaniel PA (1994). Renal 11ß-hydroxysteroid dehydrogenase activity is enhanced by ramipril and captopril. *J. Clin. Endocrinol. Metab.* 78: 830-834.

Robinson J, Canavan JP, El Haj AJ & Goldspink DF (1988). Maternal Diabetes in Rats I. Effects on placental growth and protein turnover. *Diabetes* 37: 1665-1670.

Robinson JS, Owens JA, De Barro T, Lok F & Chidzanja S (1994). Maternal nutrition and fetal growth. *Early fetal growth and development* (eds Ward RHT, Smith SK & Donnai D) 317-334. Royal College of Obstetricians and Gynaecologists, London.

Robinson S, Walton RJ, Clark PM, Barker DJP, Hales CN & Osmond C (1992). The relation of fetal growth to plasma glucose in young men. *Diabetologia* 35: 444-446.

Rodin A, Thakkar H, Taylor N & Clayton R (1994). Hyperandrogenism in polycystic ovary syndrome: evidence of dysregulation of 11beta-hydroxysteroid dehydrogenase. *N. Engl. J. Med.* 330: 460-465.

Russell MAH, Patel VA, Feyerabend C & Cole CV (1975). Plasma nicotine levels after smoking cigarettes with high, medium and low nicotine yields. *Brit. Med. J.* 11: 414-416.

Russo D, Fraser R & Kenyon CJ (1990). Increased sensitivity to noradrenaline in glucocorticoid treated rats: the effects of indomethacin and desipramine. *J. Hypertens.* 8: 827-833.

Salassa RM, Mattox VR & Rosevear JW (1962). Inhibition of mineralocorticoid activity of liquorice by spironolactone. *J. Clin. Endocrinol.* 22: 1156-1159.

Sanchis R, Sancho-Tello M & Guerri C (1986). The effects of chronic alcohol consumption on pregnant rats and their offspring. *Alcohol & Alcoholism* 21: 295-305.

Sapolsky RM (1985). Stress-induced suppression of testicular function in the wild baboon: role of glucocorticoids. *Endocrinology* 116: 2273.

Schleimer RP (1991). Potential regulation of inflammation in the lung by local metabolism of hydrocortisone. *Am. J. Resp. Cell Mol.* 4: 166-173.

Schneider H, Panigel M & Dancis J (1972). Transfer across the perfused human placenta of antipyrine, sodium and leucine. *Am. J. Obstet. Gynecol.* 114(6): 822-828.

Schneider H (1991). The role of the placenta in nutrition of the human fetus. *Am. J. Obstet. Gynecol.* 164: 967-973.

Schneider H & Huch A (1985). Dual in vitro perfusion of an isolated lobe of human placenta: method and instrumentation. *Contr. Gynec. Obstet.* 13: 40-47.

Schreiber JR, Nakamura K & Erickson GF (1982). Rat Ovary Glucocorticoid Receptor: Identification and Characterization. *Steroids* 39: 569-584.

Scott EM, McGarrigle HHG & Lachelin GCL (1990). The increase in plasma and saliva cortisol levels in pregnancy is not due to the increase in corticosteroid-binding globulin levels. *J. Clin. Endocrinol. Metab.* 71: 639-644.

Seckl JR (1994). Glucocorticoids and small babies. *Q. J. Med.* 87: 259-262.

Seckl JR & Brown RW (1994). 11-beta-hydroxysteroid dehydrogenase: on several roads to hypertension. *J. Hypertens.* 12: 105-112.

Seidman DS, Laor A, Gale R, Stevenson DK, Mashiach S & Danon YL (1991). Birth weight, current body weight, and blood pressure in late adolescence. *Brit. Med. J.* 302: 1235-1237.

Seki K, Makimura N, Mitsui C & Nagata I (1993). Osteocalcin levels in maternal and cord blood. *Obstet. Gynecol.* 81: 189-190.

Semafuko WE, Sheff MF, Grimes CA, Latif SA, Sadaniantz A, Levinson P & Morris DJ (1993). Inhibitors of 11ß-hydroxysteroid dehydrogenase and 5ß-

reductase in urine from patients with congestive heart failure. *Clin. Lab. Sci.* 23: 456-461.

Shalhoub V, Conlon D, Tassinari M, Quinn C, Partridge N, Stein GS & Lian JB (1992). Glucocorticoids promote development of the osteoblast phenotype by selectively modulating expression of cell growth and differentiation associated genes. *J Cell Biochem* 50: 425-440.

Shima M, Seino Y, Tanaka Y & et al. (1985). Bone gamma -carboxyglutamic acid containing protein in the perinatal period. *Acta Paediatr Scand* 74: 674-677.

Sidhu RK (1987). Corticosteroids in pregnancy. *Drugs and Pregnancy; Human Teratogenesis and Related Problems* (ed Hawkins DF), 2nd ed: 166-179. Edinburgh, Churchill Livingstone.

Siebe H, Baude G, Lichtenstein I, Wang D, Buhler H, Hoyer GA & Hierholzer K (1993). Metabolism of dexamethasone: sites and activity in mammalian tissues. *Renal Physiol. Bioch.* 16: 79-88.

Slotkin TA, Lappi SE, McCook EC, Tayyeb MI, Eylers JP & Seidler FJ (1992a). Glucocorticoids and the development of neuronal function: Effects of prenatal dexamethasone exposure on central noradrenergic activity. *Biol. Neonate* 61: 326-336.

Slotkin TA, Seidler FJ, Kavlock RJ & Gray JA (1992b). Fetal dexamethasone exposure accelerates development of renal function: Relationship to dose, cell differentiation and growth inhibition. *J. Dev. Physiol.* 17: 55-61.

Smith MA, Thomford PJ, Mattison DR & Slikker W, Jr. (1988). Transport and metabolism of dexamethasone in the dually perfused human placenta. *Reprod. Toxicol.* 2: 37-43.

Speiser PW, Riddick LM, Martin K & New MI (1993). Investigation of the mechanism of hypertension in apparent mineralocorticoid excess. *Metabolism Clinical and Experimental* 42: 843-845.

Spira A, Spira N, Goujrd J & et al. (1975). Smoking during pregnancy and placental weight: a multivariant analysis of 3759 cases. *J. Perinat. Med.* 3: 237-241.

Stalmans W & Laloux M (1979). Glucocorticoids and hepatic glycogen metabolism. *Glucocorticoid Hormone Action* (eds Baxter JD & Rousseau GG) 535-546. Springer Verlag, New York.

Starkman MN, Schteingart DE & Schork MA (1986). Cushing's syndrome after treatment: changes in cortisol and ACTH levels, and amelioration of the depressive syndrome. *Psychiatry Res.* 19: 177-188.

Stein ZA, Susser MV & Saenger G (1975). Famine and human development. *The Dutch Hunger Winter of 1944-45* 119-148. New York, Oxford University Press.

Sternberger LA, Hardy PH, Cuculis JJ & Meyer HG (1970). The unlabelled antibody-enzyme method of immunohistochemistry. Preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-antihorseradish peroxidase) and its use in identification of spirochetes. *Journal of Histochemistry and Cytochemistry* 18: 315-333.

Stewart PM, Wallace AM, Valentino R, Burt D, Shackleton CHL & Edwards CRW (1987). Mineralocorticoid activity of liquorice: 11-beta-hydroxysteroid dehydrogenase deficiency comes of age. *Lancet* ii: 821-823.

Stewart PM, Corrie JET, Shackleton CHL & Edwards CRW (1988). Syndrome of apparent mineralocorticoid excess. A defect in the cortisol-cortisone shuttle. *J. Clin. Invest.* 82: 340-349.

Stewart PM, Shackleton CHL, Beastall GH & Edwards CRW (1990a). 5alpha-reductase activity in polycystic ovarian syndrome. *Lancet* 335: 431-433.

Stewart PM, Wallace AM, Atherden SM, Shearing CH & Edwards CRW (1990b). Mineralocorticoid activity of carbenoxolone: contrasting effects of carbenoxolone and liquorice on 11 beta-hydroxysteroid dehydrogenase activity in man. *Clin. Sci.* 78: 49-54.

Stewart PM, Whorwood CB, Valentino R, Burt D, Sheppard MC & Edwards CRW (1993). 11-Beta-hydroxysteroid dehydrogenase activity and gene expression in the hypertensive Bianchi-Milan rat. *J. Hypertens.* 11: 349-354.

Stewart PM, Murry BA & Mason JI (1994a). Type 2 11beta -hydroxysteroid dehydrogenase in human fetal tissues. *J. Clin. Endocrinol. Metab.* 78: 1529-1532.

Stewart PM, Murry BA & Mason JI (1994b). Human kidney 11beta -hydroxysteroid dehydrogenase is a high affinity nicotinamide adenine dinucleotide-dependent enzyme and differs from the cloned type I isoform. *J. Clin. Endocrinol. Metab.* 79: 480-484.

Straus DS, Ooi GT, Orlowski CC & Rechler MM (1991). Expression of the genes for insulin-like growth factor-I (IGF-I), IGF- II, and IGF-binding proteins-1 and -2 in fetal rat under conditions of intrauterine growth retardation caused by maternal fasting. *Endocrinology* 128: 518-525.

Subramaniam M, Colvard D, Keeting PE, Rasmussen K, Riggs BL & Spelsberg TC (1992). Glucocorticoid regulation of alkaline phosphatase, osteocalcin, and

proto-oncogenes in normal human osteoblast like cells. *J Cell Biochem* 50: 411-424.

Surani MA, Kothary R, Allen ND, Singh PB, Fundele R, FergusonSmith AC & Barton SC (1990). Genome imprinting and development in the mouse. *Development* 110: 89-98.

Sweat ML, Grosser BI, Berliner DL, Swim HE, Nabors CJ, Jr. & Dougherty TF (1958). *Biochim. Biophys. Acta* 28: 591.

Sybulski S & Maughan GB (1971). Use of Streptozotocin as Diabetic Agent in Pregnant Rat. *Endocrinology* 89: 1537-1540.

Taeusch HW, Jr. (1975). Glucocorticoid prophylaxis for respiratory distress syndrome: a review of potential toxicity. *J. Pediatr.* 87: 617-623.

Takeda Y, Yoneda T, Miyamori I, Gathiram P & Takeda R (1993). 11ß-Hydroxysteroid dehydrogenase activity in mesenteric arteries of Spontaneously Hypertensive Rats. *Clin. Exp. Pharmacol. Physiol.* 20: 627-631.

Takeda Y, Miyamori I, Yoneda T, Hatakeyama H, Iki K & Takeda R (1994a). Decreased activity of 11beta -hydroxysteroid dehydrogenase in mesenteric arteries of Dahl salt-sensitive rats. *Life Sciences* 54: 1343-1349.

Takeda Y, Miyamori I, Yoneda T, Hatakeyama H, Iki K & Takeda R (1994b). Gene expression of 11ß-hydroxysteroid dehydrogenase in the mesenteric arteries of genetically hypertensive rats. *Hypertension* 23: 577-580.

Takeda Y, Miyamori I, Yoneda T, Ito Y & Takeda R (1994c). Expression of 11ß-hydroxysteroid dehydrogenase mRNA in rat vascular smooth muscle cells. *Life Sci.* 54: 281-285.

Tangalakis K, Lumbers ER, Moritz KM, Towstoless MK & Wintour EM (1992). Effect of cortisol on blood pressure and vascular reactivity in the ovine fetus. *Exp. Physiol.* 77: 709-717.

Tanner JM, Healy MJR & Lockhart RD (1956). Aberdeen growth study I: the prediction of adult body measurement from measurements taken each year from birth to 5 years. *Arch. Dis. Child.* 31: 372-381.

Tannin GM, Agarwal AK, Monder C, New MI & White PC (1991). The human gene for 11 beta-hydroxysteroid dehydrogenase. Structure, tissue distribution, and chromosomal localization. *J. Biol. Chem.* 266: 16653-16658.

Tanswell AK, Worthington D & et al. (1977). Human amniotic membrane corticosteroid 11-oxidoreductase activity. *J. Clin. Endocrinol. Metab.* 45: 721.

Teelucksingh S, Mackie ADR, Burt D, McIntyre MA, Brett LP & Edwards CRW (1990). Potentiation of hydrocortisone activity in skin by glycyrrhetinic acid. *Lancet* 335: 1060-1063.

Teelucksingh S, Benediktsson R, Lindsay RS, Burt D, Seckl JR, Edwards CRW, Nan CL & Kelly R (1991a). Liquorice. *Lancet* 337: 1549.

Teelucksingh S, Padfield PL, Tibi L, Gough KJ & Holt PR (1991b). Inhaled corticosteroids, bone formation, and osteocalcin. *Lancet* 338: 60-61.

Tipton KF (1992). Principles of enzyme assay and kinetic studies. *Enzyme Assays. A Practical Approach* (eds Eisenthal R & Danson MJ) 1st ed: 1-58. Oxford, IRL Press at Oxford University Press.

Tonolo G, Fraser R, Connell JMC & Kenyon CJ (1988). Chronic low-dose infusions of dexamethasone in rats: effects on blood pressure, body weight and plasma atrial natriuretic peptide. *J. Hypertens.* 6: 25-31.

Ulick S, Levine LS, Gunczler P, Zanconato G, Ramirez LC, Rauh W, Rosler A, Bradlow HL & New MI (1979). A syndrome of apparent mineralocorticoid excess associated with defects in the peripheral metabolism of cortisol. *J. Clin. Endocrinol. Metab.* 49: 757-764.

Ulick S, Chan CK, Rao KN, Edassery J & Mantero F (1989). A new form of the syndrome of apparent mineralocorticoid excess. *J. Steroid Biochem.* 32: 209-212.

Ulick S, Tedde R & Mantero F (1990). Pathogenesis of the type 2 variant of the syndrome of apparent mineralocorticoid excess. *J. Clin. Endocrinol. Metab.* 70: 200-206.

Urrusti J, Yoshida P, Velasco L, Frenk S, Rosado A, Sosa A, Morales M, Yoshida T & Metcoff J (1972). Human fetal growth retardation. I. Clinical features of sample with intrauterine growth retardation. *Pediatrics* 50: 547-558.

Veldhuis JD, Iranmanesh A, Johnson ML & et al (1990). Amplitude, but not frequency, modulation of adrenocorticotropin secretory bursts gives rise to the nyctohemeral rhythm of the corticotropic axis in man. *J. Clin. Endocrinol. Metab.* 71: 452-463.

Villar J & Belizan JM (1982). The timing factor in the pathophysiology of the intrauterine growth retardation syndrome. *Obstet. Gynecol. Surv.* 37: 499-506.

Virgin CE, Ha TPT, Packan DR, Tombaugh GC, Yang SH, Horner HC & Sapolsky RM (1991). Glucocorticoids inhibit glucose-transport and glutamate uptake in hippocampal astrocytes - implications for glucocorticoid neurotoxicity. *J. Neurochem.* 57: 1422-1428.

Voccia E, Saenger P, Peterson RE & et al (1979). 6ß-Hydroxycortisol excretion in hypercortisolemic states. *J. Clin. Endocrinol. Metab.* 48: 467-471.

Waddell BJ, Albrecht ED & Pepe GJ (1988a). Effect of estrogen on the metabolism of cortisol and cortisone in the baboon fetus at midgestation. *Biol. Reprod.* 38: 1006-1011.

Waddell BJ, Albrecht ED & Pepe GJ (1988b). Metabolism of cortisol and cortisone in the baboon fetus at midgestation. *Endocrinology* 122: 84-88.

Walker BR, Stewart PM, Padfield PL & Edwards CRW (1991a). Increased vascular sensitivity to glucocorticoids in essential hypertension: 11ß-hydroxysteroid dehydrogenase deficiency revisited. *J. Hypertens.* 9: 1082-1083(Abstract).

Walker BR, Yau JLW, Brett LP, Seckl JR, Monder C, Williams BC & Edwards CRW (1991b). 11 beta-hydroxysteroid dehydrogenase in vascular smooth muscle and heart: implications for cardiovascular responses to glucocorticoids. *Endocrinology* 129: 3305-3312.

Walker BR (1992). Tissue sensitivity to glucocorticoids in hypertension. 82-83.(Thesis)

Walker BR, Campbell JC, Fraser R, Stewart PM & Edwards CRW (1992a). Mineralocorticoid excess and inhibition of 11ß-hydroxysteroid dehydrogenase in patients with ectopic ACTH syndrome. *Clin. Endocrinol.* 37: 483-492.

Walker BR, Campbell JC, Williams BC & Edwards CRW (1992b). Tissue-specific distribution of the NAD(+)-dependent isoform of 11 beta-hydroxysteroid dehydrogenase. *Endocrinology* 131: 970-972.

Walker BR, Connacher AA, Webb DJ & Edwards CRW (1992c). Glucocorticoids and blood pressure: a role for the cortisol/cortisone shuttle in the control of vascular tone in man. *Clin. Sci.* 83: 171-178.

Walker BR, Aggarwal I, Williamson PM, Brown MA, Whitworth JA & Edwards CRW (1993). Defining the role of endogenous inhibitors of 11ß-dehydrogenase. *J. Endocrinol.* 137(Suppl): OC36(Abstract).

Walker BR, Aggarwal I, Stewart MJ, Padfield PL & Edwards CRW (1994). Excretion of urinary inhibitors of 11ß-dehydrogenase does not correlate with in vivo indices of enzyme activity and is not elevated in essential hypertension. *J. Endocrinol.* 104(Suppl): P8(Abstract).

Walker BR & Williams BC (1992). Corticosteroids and vascular tone: mapping the messenger maze. *Clin. Sci.* 82: 597-605.

Wang C & Leung A (1989). Glucocorticoids Stimulate Plasminogen Activator Production by Rat Granulosa Cells. *Endocrinology* 124: 1595-1601.

Waterman MR & Simpson ER (1989). Regulation of steroid hydroxylase gene expression is multifactorial in nature. *Recent Prog. Horm. Res.* 45: 533-563.

Weitzman ED, Fukushima DK, Nogeire C & et al (1971). Twenty-four hour pattern of the episodic secretion of cortisol in normal subjects. *J. Clin. Endocrinol. Metab.* 33: 14-22.

Werder E, Zachmann M & Vollmin JA (1974). Unusual steroid excretion in a child with low renin hypertension. *Res. Steroids* 6: 385-389.

White PC, Curnow KM & Pascoe L (1994). Disorders of steroid 11ß-hydroxylase enzymes. *Endocr. Rev.* 15: 421-438.

Whitworth JA, Gordon D, McLachlan-Troup N, Scoggins BA & Moulds RWF (1989a). Dexamethasone suppression in essential hypertension: effects on cortisol and blood pressure. *Clin. Exp. Hypertens. A.* II: 323-335.

Whitworth JA, Stewart PM, Burt D, Atherden SM & Edwards CRW (1989b). The kidney is the major site of cortisone production in man. *Clin. Endocrinol.* 31: 355-361.

Whorwood CB, Franklyn JA, Sheppard MC & Stewart PM (1992). Tissue localization of 11 beta-hydroxysteroid dehydrogenase and its relationship to the glucocorticoid receptor. *J. Steroid Biochem. Molec. Biol.* 41: 21-28.

Whorwood CB, Barber PC, Gregory J, Sheppard MC & Stewart PM (1993a). 11beta -Hydroxysteroid dehydrogenase and corticosteroid hormone receptors in the rat colon. *American Journal of Physiology - Endocrinology and Metabolism* 264: E951-E957.

Whorwood CB, Sheppard MC & Stewart PM (1993b). Tissue specific effects of thyroid hormone on 11beta -hydroxysteroid dehydrogenase gene expression. *J. Steroid Biochem. Molec. Biol.* 46: 539-547.

Wier PJ & Miller RK (1985). Oxygen transfer as an indicator of perfusion variability in the isolated human placental lobule. *Contr. Gynec. Obstet.* 13: 127-131.

Wigglesworth JS (1989). Aetiology of fetal undergrowth. *Fetal Growth* (eds Sharp F, Fraser RB & Milner RDG) 185-195. London, Springer Verlag.

Williamson PM, Brown MA, Walker BR, Edwards CRW & Whitworth JA (1994). 11ß-dehydrogenase activity and its inhibitors in preeclampsia. *J. Hypertens.* 12(Suppl): 1043(Abstract).

Yau JLW, Van Haarst AD, Moisan MP, Fleming S, Edwards CRW & Seckl JR (1991). 11 beta-Hydroxysteroid dehydrogenase mRNA expression in rat kidney. *Am. J. Physiol.* 260: F764-F767.

Young MP & Schneider H (1984). Metabolic integrity of the isolated perfused lobule of human placenta. *Placenta* 5: 95-104.

transferrable elements that do not resemble conventional plasmids[5] have been isolated from these TriR/MuR *S aureus* transcipients, and attempts to clone the resistance are underway. There is no cross-resistance to propamidine, benzalkonium chloride, cetrimide, hexachlorophane, chlorhexidine acetate, chlorhexidine gluconate, or 9-aminoacridine. Our initial experiments indicate that the presence of TriR does not influence the rate of kill after 30 s to 2 min of exposure to triclosan. Similar experiments have suggested that the same is true for MRSA with low-level chlorhexidine resistance.[4]

However, the possibility of a rise in MIC to triclosan should be considered if eradication of MRSA is difficult with this agent, and we would be grateful if such strains were sent to the division of hospital infection for further study.

Division of Hospital Infection,
Central Public Health Laboratory,
London NW9 5HT, UK

B. D. COOKSON
H. FARRELLY
P. STAPLETON

Department of Microbiology,
Warrington District Hospital

R. P. J. GARVEY
M. R. PRICE

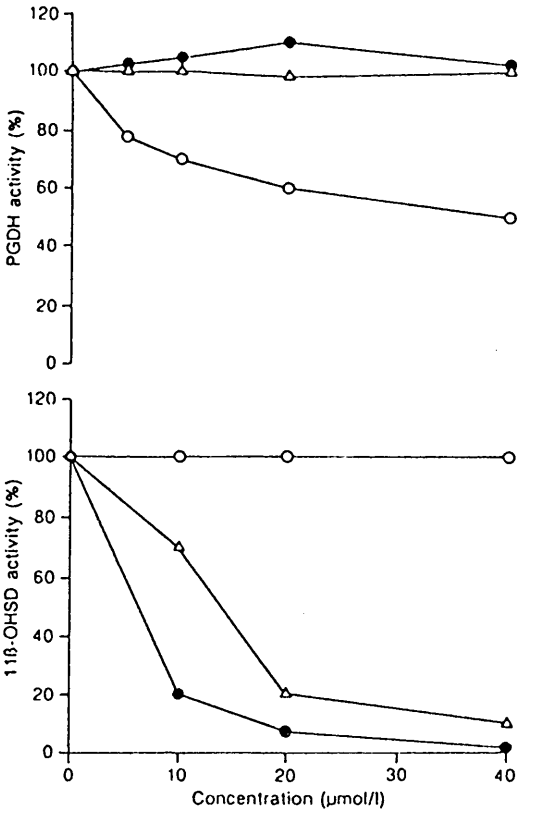
1. Brumfitt W, Dixson S, Hamilton-Miller JMT. Resistance to antiseptics in methicillin and gentamicin resistant *Staphylococcus aureus*. *Lancet* 1985; i: 1442–43.
2. Yamomoto T, Tamura Y, Yokota T. Antiseptic and antibiotic resistance plasmid in *Staphylococcus aureus* that possesses ability to confer chlorhexidine and acrinol resistance. *Antimicrob Agents Chemother* 1988; 32: 932–35.
3. Tennent JA, Lyon BR, Midgley M, Gwyn Jones I, Purewal AS, Skurray R. Physical and biochemical characterisation of the *qacA* gene encoding antiseptic and disinfectant resistance in *Staphylococcus aureus*. *J Gen Microbiol* 1989; 135: 1–10.
4. Cookson BD, Bolton M, Platt J, Phillips I. Chlorhexidine resistance in methicillin-resistant *S aureus* or just an elevated MIC? An in vitro and in vivo assessment. Fourth European Congress in Clinical Microbiology, Nice, 1989: abstract 476.
5. Rahman M, Connely S, Noble WC, Cookson BD, Phillips I. The diversity of staphylococci exhibiting high-level resistance to mupirocin. *J Med Microbiol* 1990; 33: 97–100.

Liquorice

SIR,—Dr Baker and Dr Fanestil (Feb 16, p 428) point out the structural homology between the prostaglandin metabolising enzyme, 15-hydroxyprostaglandin dehydrogenase (PGDH) and 11 β-hydroxysteroid dehydrogenase (11β-OHSD), which converts cortisol to its inactive product cortisone. We have previously shown that liquorice derivatives are potent inhibitors of 11β-OHSD. Baker and Fanestil speculate that by inhibiting PGDH, liquorice might, in part, exert its biological effects through prostaglandins. Their suggestion is based on data from Peskar et al[1] who showed inhibition of gastric prostaglandin metabolism by high concentrations of carbenoxolone.

Liquorice and its derivatives possess both mineralocorticoid and glucocorticoid properties. The mechanism of these actions was poorly understood, although the presence of an intact adrenal gland or cortisol replacement in patients with adrenal insufficiency was known to be essential.[2,3] We found that glycyrrhetinic acid, the active component of liquorice, is a potent inhibitor of 11β-OHSD in vitro; in-vivo studies showed that this inhibition could account for both the mineralocorticoid and glucocorticoid effects of liquorice.[4,5] To assess whether altered prostaglandin metabolism, as suggested by Baker and Fanestil, might contribute to these biological actions, we examined the effects of glycyrrhetinic acid and carbenoxolone on PGDH and 11β-OHSD activity in vitro. Human placental tissue was chosen because it is a rich source of both 11β-OHSD and PGDH.

Chorionic homogenate (protein content 1·5 mg) was incubated with 500 ng PGE_2 and 1 mmol/l NAD at 37°C for 15 min in a phosphate buffer containing 20% glycerol (pH 8·4). PGDH activity was measured by radioimmunoassay of the quantity of 15-keto-PGE_2 produced after transformation to its methyl oxime. The experiment was completed with glycyrrhetinic acid, carbenoxolone, and 5-(4-fluorobenzoyl)2-hydroxybenzene acetic acid (FHBA), a known PGDH inhibitor. Substances were tested at concentrations up to 40 μmol/l. The effects of similar concentrations of these substances on human placental 11β-OHSD were evaluated as previously described.[4] Briefly, 0·3 mg protein from fresh placental homogenate was incubated with 12 mmol/l ^{3}H-cortisol and 200 μmol/l NAD in Krebs-Ringer buffer at 37°C for 1 h. Steroids were extracted with ethyl acetate and separated by thin-layer chromatography. Activity of 11β-OHSD was expressed as a percentage conversion of ^{3}H-cortisol to ^{3}H-cortisone. The results show that glycyrrhetinic acid and carbenoxolone had no effect on PGDH activity, in contrast to the inhibition produced by FHBA (figure, upper). FHBA had no effect on 11β-OHSD activity whereas carbenoxolone and glycyrrhetinic acid were inhibitory (figure, lower).



Effects of liquorice on enzymes of prostaglandin and steroid metabolism.

Upper, effects of glycyrrhetinic acid, carbenoxolone, and FHBA on 15-hydroxyprostaglandin dehydrogenase activity expressed as percentage of control; lower, effects of the same substances on 11β-hydroxysteroid dehydrogenase. ●, glycyrrhetinic acid; □, carbenoxolone; ○, FHBA.

Liquorice ingestion has been shown to produce mineralocorticoid effects even when plasma glycyrrhetinic acid concentrations are below 2 μmol/l, the lower limit of detection of the assay.[5] This evidence, together with the current findings, makes it unlikely that altered prostaglandin metabolism contributes to the salt-retaining or anti-inflammatory actions of liquorice and its derivatives.

Department of Medicine,
Western General Hospital,
Edinburgh EH4 2XU, UK

S. TEELUCKSINGH
R. BENEDIKTSSON
R. S. LINDSAY
D. BURT
J. R. SECKL
C. R. W. EDWARDS

MRC Reproductive Biology Unit,
Edinburgh

CHENG LI NAN
R. KELLY

1. Peskar BM, Holland A, Peskar BA. Effect of carbenoxolone on prostaglandin synthesis and degradation. *J Pharm Pharmacol* 1976; 28: 146–48.
2. Borst JGG, Ten Holt SP, De Vries LA, Molhuysen JA. Synergistic action of liquorice and cortisone in Addison's and Simmond's disease. *Lancet* 1953; i: 657–63.
3. Elmadjian F, Hope JM, Pincus G. The action of mono-ammonium glycyrrhizinate on adrenalectomised subjects and its synergism with hydrocortisone. *J Clin Endocrinol Metab* 1956; 16: 338–49.
4. Teelucksingh S, Mackie ADR, Burt D, McIntyre MA, Brett L, Edwards CRW. Potentiation of hydrocortisone activity in skin by glycyrrhetinic acid. *Lancet* 1990; 335: 1060–63.
5. Stewart PM, Valentino R, Wallace AM, Burt D, Shackleton CHL, Edwards CRW. Mineralocorticoid activity of liquorice: 11β-hydroxysteroid deficiency comes of age. *Lancet* 1987; ii: 821–24.

11β-Hydroxysteroid dehydrogenase in the rat ovary: high expression in the oocyte

R. Benediktsson, J. L. W. Yau, S. Low, L. P. Brett*, B. E. Cooke†, C. R. W. Edwards and J. R. Seckl

Departments of Medicine and *Pathology, University of Edinburgh, Western General Hospital, Edinburgh EH4 2XU, U.K.

†Department of Biochemistry, Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2PF, U.K.

(Requests for offprints should be addressed to J. R. Seckl)

RECEIVED 24 January 1992

ABSTRACT

The enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) catalyses the conversion of physiological glucocorticoids to inactive products, thus modifying the access of glucocorticoids to glucocorticoid and mineralocorticoid receptors. Glucocorticoids may affect ovarian function both indirectly and via binding to ovarian receptors. We have demonstrated 11β-HSD bioactivity and mRNA expression in rat ovary *in vitro*. The enzyme was localized to oocytes and luteal bodies immunohistochemically using two antibodies raised against purified rat liver 11β-HSD. These data are supported by in-situ hybridization studies, which also localized 11β-HSD mRNA expression to oocytes and luteal bodies. The results suggest that 11β-HSD may modulate the effects of glucocorticoid on ovarian function.

Journal of Endocrinology (1992) **135,** 53–58

INTRODUCTION

Glucocorticoid hormones are important regulators of cell function in many tissues. Recent evidence suggests that glucocorticoids affect ovarian physiology. Thus systemic administration of exogenous glucocorticoids has been shown to reduce ovarian weight (Inazu, Iwata & Satoh, 1990) and to inhibit ovulation (Baldwin & Sawyer, 1974). Although the inhibition of ovulation is thought to reflect glucocorticoid actions at the anterior pituitary, where they inhibit the preovulatory luteinizing hormone (LH) surge (Baldwin & Sawyer, 1974), the presence of glucocorticoid receptors (Schreiber, Nakamura & Erickson, 1982) and glucocorticoid-responsive gene products (Malbon & Hadcock, 1988; Albiston, Lock, Burger & Krozowski, 1990) in the ovary suggests that direct effects also occur. In support of this, glucocorticoids have been shown to inhibit follicle-stimulating hormone (FSH)-stimulated aromatase activity (Hsueh & Erickson, 1978) and to augment progesterone accumulation (Adashi, Jones & Hsueh, 1981) in granulosa cells *in vitro*. Similarly, glucocorticoids stimulate the production of plasminogen activator by isolated granulosa cells (Wang & Leung, 1989; Jia & Hsueh, 1990), an action thought to be important in the control of ovulation (Reich, Miskin & Tsafriri, 1985).

Both protein purification and more recent molecular cloning studies have demonstrated the existence of two types of glucocorticoid receptor, type-I (high-affinity or mineralocorticoid receptor) and type-II (low-affinity or glucocorticoid receptor). Although the type-I and -II receptors show well-described hierarchies of affinity for the various physiological and synthetic glucocorticoids *in vitro*, ligand access to receptors in many tissues *in vivo* is regulated by 11β-hydroxysteroid dehydrogenase (11β-HSD). This microsomal enzyme catalyses the reversible conversion of physiological glucocorticoids (cortisol in man, corticosterone in the rat) to inactive products (cortisone or 11-dehydrocorticosterone). The 11-dehydro products have very low affinities for either type-I or type-II receptors. The activity of 11β-HSD appears to be responsible for the in-vivo aldosterone selectivity of the otherwise non-selective renal type-I receptor (Edwards, Stewart, Burt *et al.* 1988; Funder, Pearce, Smith & Smith, 1988) and modulates glucocorticoid access to type-II receptors in the skin (Teelucksingh,

Journal of Endocrinology (1992) **135,** 53–58 *Printed in Great Britain*
0022-0795/92/0135-0053 $02.00/0

Mackie, Burt *et al.* 1990) and cerebellum (Moisan, Seckl, Brett *et al.* 1990*a*). Within reproductive tissues, 11β-HSD is present in the placenta where it is thought to protect the fetus from the deleterious effects of exposure to maternal glucocorticoids (Beitins, Bayard, Ances *et al.* 1973; López Bernal, Anderson & Turnbull, 1982) and the enzyme has been demonstrated in the testis where it may regulate Leydig cell–glucocorticoid interactions which are critical in the control of testosterone production and the onset of puberty (Phillips, Lakshmi & Monder, 1989).

By analogy with the testis, the ovary might be expected to contain 11β-HSD which could regulate glucocorticoid access to receptors and thus modulate corticosteroid effects *in vivo*. Although previous studies have not found 11β-HSD bioactivity in the rat ovary (Ghraf, Vetter, Zandveld & Schriefers, 1975), human ovaries have been shown to be able to convert cortisol to cortisone (Murphy, 1981*a*), and to contain mRNA encoding 11β-HSD (Tannin, Agarwal, Monder *et al.* 1991). Therefore, we have re-examined whether 11β-HSD bioactivity and mRNA expression are present in the ovary of the rat, and have determined their tissue localization.

MATERIALS AND METHODS

11β-HSD bioassay

Dehydrogenase component

Ovaries, obtained from adult female Wistar rats (240 g; $n = 3$–4) in pro-oestrus, were homogenized in Krebs–Ringer bicarbonate buffer (118 mmol NaCl/l, 3·8 mmol KCl/l, 1·19 mmol KH_2PO_4/l, 2·54 mmol $CaCl_2 \cdot 2H_2O$/l, 1·19 mmol $MgSO_4 \cdot 7H_2O$/l, 25 mmol $NaHCO_3$/l) using a Dounce tissue grinder. The total protein content was estimated colorimetrically (Bio-Rad protein assay kit, Hemel Hempstead, Herts, U.K.), using a sample of the homogenate. The incubation was carried out in duplicate with 0·0625 g, 0·25 g and 0·5 g protein/l and a final concentration of 200 μmol NAD/l and 12 nmol [1,2,6,7-^{3}H]corticosterone/l (specific activity: 84 Ci/mmol; Amersham International plc, Little Chalfont, Bucks, U.K.) in Krebs–Ringer buffer (+0·2% bovine serum albumin and glucose) for 10, 20 and 60 min at 37 °C. The total volume was 250 μl. Rat kidney, known to have very high 11β-HSD bioactivity, was used as a positive control (at 0·25 g protein/l for 10 min), whilst incubation of buffer alone provided an assay blank. After incubation, steroids were extracted with ethyl acetate and separated by high-pressure liquid chromatography (HPLC). The percentage conversion of [^{3}H]corticosterone to [^{3}H]11-dehydrocorticosterone was calculated from the radioactivity of each fraction. Results are expressed as means ± S.E.M.

Reductase component

For preparation of [^{3}H]11-dehydrocorticosterone, [^{3}H]corticosterone (Amersham) was incubated with rat kidney homogenate for 2 h at 37 °C in the presence of 200 μmol NAD/l. After incubation, the steroids were extracted with ethyl acetate and separated by thin-layer chromatography. Extraction yield was 42% and purity > 93% as assessed by HPLC. Ovarian homogenate was prepared as above ($n = 3$), and 0·25 g protein/l incubated with 12 nmol [^{3}H]11-dehydrocorticosterone/l for 10 min at 37 °C in the presence of 200 μmol NADH/l in Krebs–Ringer buffer as above. Steroids were separated and assayed by HPLC as for dehydrogenase activity.

Northern analysis

Ovary, hippocampus, kidney and liver were rapidly removed from female Wistar rats (240 g) after cervical dislocation, snap-frozen and stored at −85 °C. Total RNA was extracted from each tissue by the acid guanidinium thiocyanate method, as previously described (Chomczynski & Sacchi, 1987). Approximately 20 μg ovary, 15 μg hippocampus and 10 μg kidney and liver total RNA were fractionated on a 1·2% agarose–0·7 mol formaldehyde gel/l and blotted on to nitrocellulose (Hybond C extra; Amersham International plc) by capillary transfer overnight. Hybridization was performed at 42 °C overnight in 50% formamide with a randomly primed ^{32}P-labelled 11β-HSD cDNA probe cloned from a rat liver cDNA library (Agarwal, Monder, Eckstein & White, 1989), consisting of the excised p11DH insert. The membrane was washed to a final stringency of 0·2 × SSC (1 × SSC equals 0·5 mol NaCl and 0·015 mol sodium citrate per litre), 0·1% sodium dodecyl sulphate at 60 °C and exposed to Kodak XAR film for 2 days as previously described (Moisan, Seckl & Edwards, 1990*b*).

Immunohistochemistry

Adult Wistar rats (240 g) were deeply anaesthetized with pentobarbitone and perfused through the ascending aorta with 250 ml ice-cold saline followed by 500 ml ice-cold paraformaldehyde (4 mol/l) in phosphate buffer (0·1 mol/l, pH 7·4). Ovaries were removed and post-fixed overnight. As previously described (Moisan *et al.* 1990*a*) sections (4 μm) were cut and immunostained using two separate polyclonal rabbit antisera (5–125 at 1:50 dilution and 5–126 at 1:100 dilution) raised against purified rat liver 11β-HSD as described elsewhere (Monder & Lakshmi, 1990). Detection was by the peroxidase–antiperoxidase method (Sternberger, Hardy, Cuculis & Meyer, 1970) using reagents from Dako Ltd, High Wycombe, Bucks, U.K. Control sections were immunostained using preimmune rabbit serum.

In-situ hybridization

Adult Wistar rats (240 g) were killed by cervical dislocation, the ovaries rapidly removed and immediately frozen on dry ice. Cryostat sections (10 μm) were mounted on to gelatin- and poly-L-lysine-coated microscope slides and stored at −85 °C. Tissue sections were post-fixed in 4% paraformaldehyde/phosphate (0·1 mol/l) buffer and washed in three changes of 2 × SSC containing 0·02% diethylpyrocarbonate. T3 RAN polymerase (Promega Ltd, Southampton, Hants, U.K.) was used to transcribe a 598 bp antisense cRNA probe containing ^{35}S-labelled UTP from Sty I-linearized pBluescript vector containing the 1265 bp 11β-HSD cDNA insert (Agarwal *et al.* 1989). The probe was denatured and added at a final concentration of 10×10^6 c.p.m./ml to hybridization buffer (50% formamide, 0·6 mol NaCl/l, 10 mmol Tris/l, pH 7·5, 0·02% Ficoll, 0·02% polyvinylpyrrolidone, 0·1% bovine serum albumin, 1 mmol EDTA/l, 0·1 g salmon sperm DNA/l, 0·1 g yeast tRNA/l, 100 g dextran sulphate/l and 10 mmol dithiothreitol/l) as previously described (Yau, Van Haarst, Moisan *et al.* 1991). In brief, 65 μl aliquots of probe were pipetted on to sections and covered with a coverslip (22 × 50 mm) which was sealed with DPX mounting medium. Slides were incubated overnight at 50 °C. After hybridization, coverslips were removed by rinsing three to four times in 2 × SSC at room temperature, sections treated with RNAase A (30 g/l, 45 min at 37 °C) (Boehringer Mannheim, Lewes, East Sussex, U.K.) and washed in reducing salt concentrations to a final stringency of 0·1 × SSC/14 mmol 2-mercaptoethanol/l at 60 °C. After dehydration in increasing concentrations of ethanol in 0·3 mol sodium acetate/l, slides were dried in air, dipped in photographic emulsion (Ilford Mobberley, Knutsford, Cheshire, U.K.) and exposed in light-tight boxes at 4 °C for 21 days before being developed (D19, Ilford) and counterstained with haematoxylin–eosin. Controls were hybridized with non-complementary 'sense' probes of the same specific activity under identical conditions.

RESULTS

11β-HSD activity

11β-HSD activity in the dehydrogenase direction was found in all ovarian homogenates and increased linearly with protein concentration up to 0·5 g/l (data not shown). The time curves for the three different protein concentrations (Text-fig. 1) indicate linearity with respect to time for up to 10 min. At a protein concentration of 0·25 g/l incubated for 10 min, ovarian activity was 10% of kidney activity (ovary, $n = 3$: 179·6 ± 30·5 fmol product formed; kidney, $n = 2$: 1742·4 ± 44·1 fmol product formed). No conversion of [^{3}H]11-dehydrocorticosterone to [^{3}H]corticosterone was found.

Northern blots

A single band hybridizing to the 11β-HSD probe was detected in ovarian total RNA which corresponded to the major 11β-HSD mRNA species demonstrated in rat liver and hippocampus. There was no evidence of expression of multiple 11β-HSD mRNA species as found in rat kidney (Text-fig. 2).

Immunohistochemistry

Using either antiserum to purified rat liver 11β-HSD, a consistently strong positive immunostaining was found in the oocyte (Pl. 1). Additionally, positive immunostaining of moderate or low intensity was detected in cells comprising the luteal bodies (not shown). This varied between the luteal masses rather than within a given luteal body, presumably reflecting their varying stages of maturation. No staining was observed in the granulosa cells, theca cells, stroma or other ovarian components (Pl. 1). No staining of any ovarian subregion was demonstrated with preimmune serum.

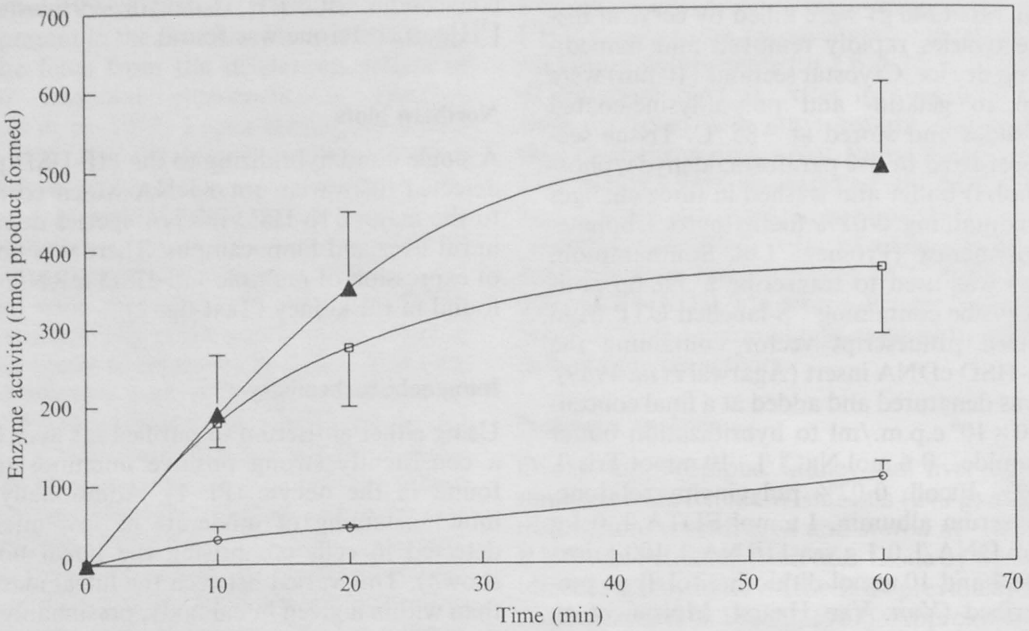
In-situ hybridization

11β-HSD mRNA expression was localized to oocytes and was also found to a variable extent over the luteal bodies, varying between luteal masses rather than within individual bodies and parallelling the immunohistochemical findings (Pl. 2).

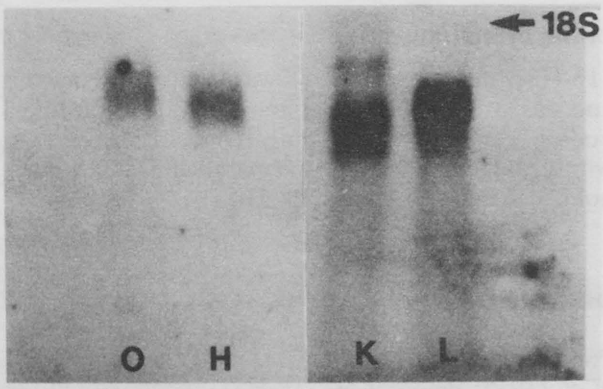
DISCUSSION

Previous studies on ovarian 11β-HSD are few. Ghraf *et al.* (1975) reported no 11β-HSD bioactivity in the rat ovary but, in contrast, Murphy (1981*a*) found considerable bioactivity in human ovaries; both studies used similar methods.

We now report high 11β-HSD bioactivity in homogenized whole ovaries, in agreement with the findings of Murphy (1981*a*). The reason for the discrepancy between our data and those of Ghraf *et al.* (1975) is unclear but may relate to the very high pH (9·5) used in the latter study. The presence of 11β-HSD bioactivity is supported by the clear expression of mRNA encoding the 'liver-type' isoform of the enzyme. Using both immunohistochemistry and in-situ hybridization we have localized 11β-HSD mRNA antigen to oocytes and, to a lesser extent, to luteal bodies. The



TEXT-FIGURE 1. 11β-Hydroxysteroid dehydrogenase bioactivity (measured as fmol [^{3}H]11-dehydrocorticosterone formed from [^{3}H]corticosterone) in whole ovarian homogenates at protein concentrations of 0·0625 g/l (○; $n=3$), 0·25 g/l (□; $n=4$) and 0·5 g/l (▲; $n=3$). Values are means ± S.E.M.



TEXT-FIGURE 2. Autoradiograph of a Northern blot of total mRNA hybridized with ^{32}P-labelled cDNA probes to 11β-hydroxysteroid dehydrogenase mRNA. O = ovary, H = hippocampus, K = kidney, L = liver. Note the presence of a single band in ovary corresponding to the one found in hippocampus and liver. Kidney shows multiple hybridizing mRNA species.

concordance of immunoreactivity and mRNA expression is in contrast to distal renal tubule (Edwards *et al.* 1988; Rundle, Funder, Lakshmi & Monder, 1989; Yau *et al.* 1991), where mRNA expression and bioactivity (Náray-Fejes-Tóth, Watlington & Fejes-Tóth, 1991) are found, but immunoreactivity (liver-type) is not detected. On the other hand, in hippocampus, cerebellum and vasculature, 11β-HSD mRNA expression colocalizes with immunoreactivity (Moisan *et al.* 1990*a*,*b*; Walker, Yau, Brett *et al.* 1991). It is thus likely that ovarian luteal bodies and oocytes express the liver-type mRNA and protein which may modulate glucocorticoid access to receptors rather than the distal convoluted tubule isoform which is completely protective of the colocalized mineralocorticoid receptor.

The interesting finding of high bioactivity localized to the oocyte itself, as indicated by in-situ hybridization and immunohistochemistry, implies modification of the oocyte glucocorticoid exposure and makes the presence of steroid receptors within the oocyte likely, as is the case in other 11β-HSD locations. Although glucocorticoids are believed to inhibit ovulation by inhibiting the preovulatory LH surge, our findings point to a possible local role of glucocorticoids in ovulation and/or oocyte differentiation. Interestingly, the specificity of 11β-HSD for 11β-hydroxyprogesterone and 11β-hydroxypregnenolone as well as their α-counterparts is much higher than for cortisol or corticosterone (Murphy, 1981*b*), raising the intriguing possibility that the substrate for ovarian 11β-HSD is not necessarily a glucocorticoid.

We were unable to find any indication of the presence of 11β-HSD in the granulosa cells, which

previously have been shown to contain glucocorticoid receptors (Schreiber *et al.* 1982) and to be metabolically regulated by glucocorticoids (Hsueh & Erickson, 1978; Adashi *et al.* 1981; Wang & Leung, 1989). This absence of 11β-HSD expression in the granulosa cells could be the result of aromatase activity generating oestrogen, which has been shown to inhibit potently activity of the liver-type 11β-HSD (Monder & Shackleton, 1984), as present in the ovary. Similarly, we did not demonstrate the presence of 11β-HSD in theca cells, although dexamethasone has been shown to alter their metabolism (reducing carbonyl reductase content) (Inazu *et al.* 1990). Our finding of 11β-HSD immunoreactivity and mRNA expression in the luteal cells but not the theca cells may be another example of cellular ontogeny, as we have shown in the skin (Teelucksingh *et al.* 1990), where 11β-HSD is not detected in the basal cell layer but is located in cells of the upper epidermis.

In summary, we have found 11β-HSD gene expression and bioactivity in the rat ovary, localized mainly in the oocyte and luteal masses. This suggests that glucocorticoids may exert a local modulatory role on ovarian function, which can be altered by cell-specific metabolism of steroids. The importance of 11β-HSD in ovarian function remains to be determined.

ACKNOWLEDGEMENTS

We thank Dr C. Monder for generously providing the 11β-HSD antisera and cDNA clone. This work was supported by a Wellcome Trust/Royal Society of Edinburgh Senior Research Fellowship (J.R.S.) and a grant from the Scottish Hospital Endowments Research Trust.

REFERENCES

Adashi, E. Y., Jones, P. B. C. & Hsueh, A. J. W. (1981). Synergistic effects of glucocorticoids on the stimulation of progesterone production by follicle-stimulating hormone in cultured rat granulosa cells. *Endocrinology* **109,** 1888–1894.

Agarwal, A. K., Monder, C., Eckstein, B. & White, P. C. (1989). Cloning and expression of rat cDNA encoding corticosteroid 11β-dehydrogenase. *Journal of Biological Chemistry* **264,** 18939–18943.

Albiston, A. L., Lock, P., Burger, H. G. & Krozowski, Z. S. (1990). Cloning and characterization of the rat α-inhibin gene. *Molecular and Cellular Endocrinology* **68,** 121–128.

Baldwin, D. M. & Sawyer, C. H. (1974). Effects of dexamethasone on LH release and ovulation in the cyclic rat. *Endocrinology* **94,** 1397–1403.

Beitins, I. Z., Bayard, F., Ances, I. G., Kowarski, A. & Migeon, C. J. (1973). The metabolic clearance rate, blood production, interconversion and transplacental passage of cortisol and cortisone in pregnancy near term. *Pediatric Research* **7,** 509–519.

Chomczynski, P. & Sacchi, N. (1987). Single-step method of RNA isolation by guanidinum thiocyanate–phenol–chloroform extraction. *Analytical Biochemistry* **162,** 156–159.

Edwards, C. R. W., Stewart, P. M., Burt, D., Brett, L. P., McIntyre, M. A., Sutanto, W. S., de Kloet, E. R. & Monder, C. (1988). Localization of 11β-hydroxysteroid dehydrogenase tissue-specific protector of the mineralocorticoid receptor. *Lancet* **ii,** 986–989.

Funder, J. W., Pearce, P. T., Smith, R. & Smith, A. L. (1988). Mineralocorticoid action: Target tissue specificity is enzyme, not receptor, mediated. *Science* **242,** 583–585.

Ghraf, R., Vetter, U., Zandveld, J. M. & Schriefers, H. (1975). Organ-specific ontogenesis of steroid hormone metabolizing enzyme activities in the rat. *Acta Endocrinologica* **79,** 192–201.

Hsueh, A. J. W. & Erickson, G. F. (1978). Glucocorticoid inhibition of FSH-induced estrogen production in cultured rat granulosa cells. *Steroids* **32,** 639–648.

Inazu, N., Iwata, N. & Satoh, T. (1990). Inhibitory effect of glucocorticoid and stimulatory effect of human chorionic gonadotropin on ovarian carbonyl reductase. *Life Sciences* **46,** 841–848.

Jia, X. C. & Hsueh, A. J. W. (1990). Synergistic effect of glucocorticoids and androgens on the hormonal induction of tissue plasminogen activator. Activity and messenger ribonucleic acid level in granulosa cells. *Molecular and Cellular Endocrinology* **68,** 143–151.

López Bernal, A., Anderson, A. B. M. & Turnbull, A. C. (1982). The lack of influence of labor on human placental 11β-hydroxysteroid dehydrogenase activity. *Journal of Clinical Endocrinology and Metabolism* **54,** 1251–1254.

Malbon, C. C. & Hadcock, J. R. (1988). Evidence that glucocorticoid response elements in the 5′-noncoding region of the hamster beta 2-adrenergic receptor gene are obligate for glucocorticoid regulation of receptor mRNA levels. *Biochemical and Biophysical Research Communications* **154,** 676–681.

Moisan, M. P., Seckl, J. R., Brett, L. P., Monder, C., Agarwal, A. K., White, P. C. & Edwards, C. R. W. (1990*a*). 11Beta-hydroxysteroid dehydrogenase messenger ribonucleic acid expression, bioactivity and immunoreactivity in rat cerebellum. *Journal of Neuroendocrinology* **2,** 853–858.

Moisan, M. P., Seckl, J. R. & Edwards, C. R. W. (1990*b*). 11β-Hydroxysteroid dehydrogenase bioactivity and messenger RNA expression in rat forebrain: Localization in hypothalamus, hippocampus, and cortex. *Endocrinology* **127,** 1450–1455.

Monder, C. & Lakshmi, M. (1990). Corticosteroid 11β-dehydrogenase of rat tissues: Immunological studies. *Endocrinology* **126,** 2435–2443.

Monder, C. & Shackleton, C. H. L. (1984). 11β-Hydroxysteroid dehydrogenase; fact or fancy? *Steroids* **44,** 383–417.

Murphy, B. E. P. (1981*a*). Ontogeny of cortisol–cortisone interconversion in human tissues: A role for cortisone in human fetal development. *Journal of Steroid Biochemistry* **14,** 811–817.

Murphy, B. E. P. (1981*b*). Specificity of human 11β-hydroxysteroid dehydrogenase. *Journal of Steroid Biochemistry* **14,** 807–809.

Náray-Fejes-Tóth, A., Watlington, C. O. & Fejes-Tóth, G. (1991). 11β-Hydroxysteroid dehydrogenase activity in the renal target cells of aldosterone. *Endocrinology* **129,** 17–21.

Phillips, D. M., Lakshmi, V. & Monder, C. (1989). Corticosteroid 11β-dehydrogenase in rat testis. *Endocrinology* **125,** 209–216.

Reich, R., Miskin, R. & Tsafriri, A. (1985). Follicular plasminogen activator: Involvement in ovulation. *Endocrinology* **116,** 516–521.

Rundle, S. E., Funder, J. W., Lakshmi, V. & Monder, C. (1989). The intrarenal localization of mineralocorticoid receptors and 11β-dehydrogenase: immunocytochemical studies. *Endocrinology* **125,** 1700–1704.

Schreiber, J. R., Nakamura, K. & Erickson, G. F. (1982). Rat ovary glucocorticoid receptor: Identification and characterization. *Steroids* **39,** 569–584.

Sternberger, L. A., Hardy, P. H. Jr., Cuculis, J. J. & Meyer, H. G. (1970). The unlabeled antibody-enzyme method of immunohistochemistry. Preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-antihorseradish peroxidase) and its use in identification of spirochetes. *Journal of Histochemistry and Cytochemistry* **18,** 315–333.

Tannin, G. M., Agarwal, A. K., Monder, C., New, M. I. & White, P. C. (1991). The human gene for 11β-hydroxysteroid dehydrogenase. Structure, tissue distribution, and chromosomal localization. *Journal of Biological Chemistry* **266,** 16653–16658.

Teelucksingh, S., Mackie, A. D. R., Burt, D., McIntyre, M. A., Brett, L. P. & Edwards, C. R. W. (1990). Potentiation of hydrocortisone activity in skin by glycyrrhetinic acid. *Lancet* **335,** 1060–1063.

Walker, B. R., Yau, J. L., Brett, L. P., Seckl, J. R., Monder, C., Williams, B. C. & Edwards, C. R. W. (1991). 11β-Hydroxysteroid dehydrogenase in vascular smooth muscle and heart: Implications for cardiovascular responses to glucocorticoids. *Endocrinology* **129,** 3305–3312.

Wang, C. & Leung, A. (1989). Glucocorticoids stimulate plasminogen activator production by rat granulosa cells. *Endocrinology* **124,** 1595–1601.

Yau, J. L. W., Van Haarst, A. D., Moisan, M. P., Fleming, S., Edwards, C. R. W. & Seckl, J. R. (1991). 11β-hydroxysteroid dehydrogenase mRNA expression in rat kidney. *American Journal of Physiology* **29,** F764–F767.

DESCRIPTION OF PLATES

Plate 1

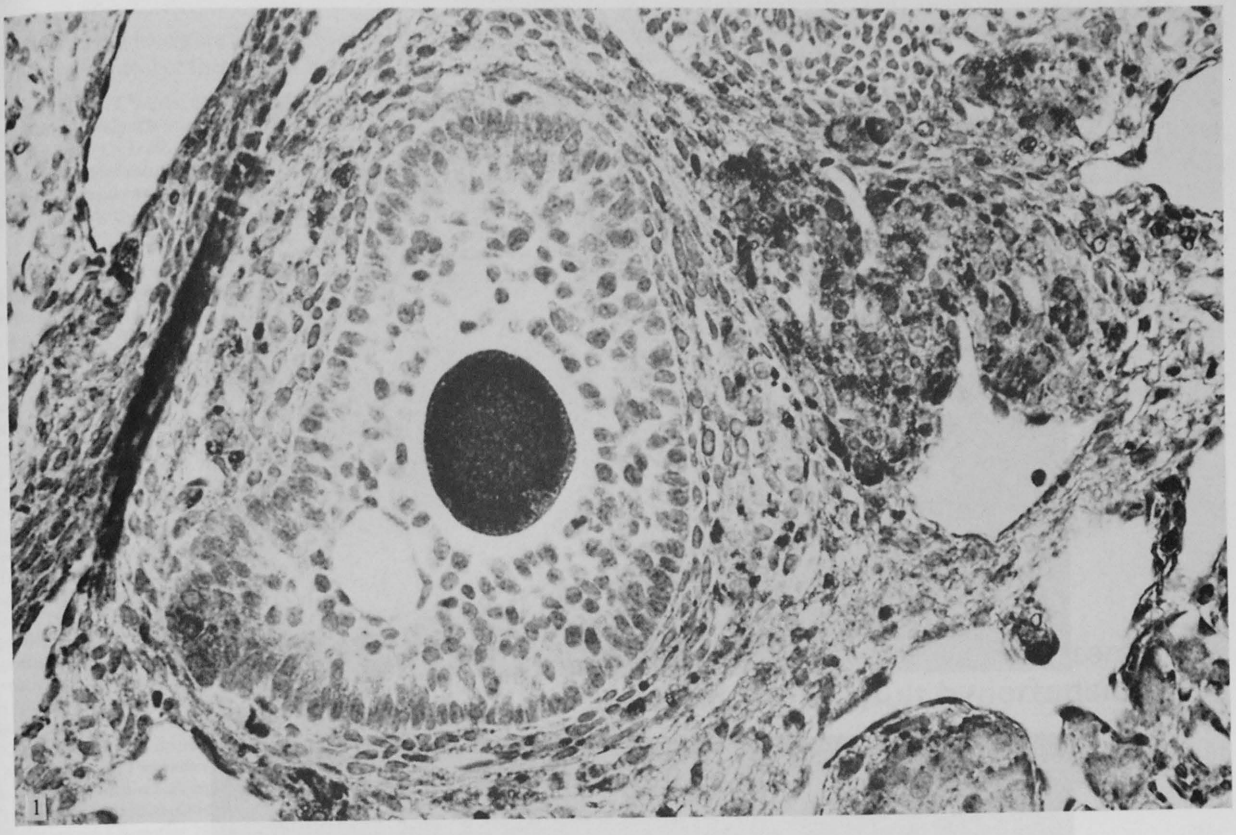
FIGURE 1. Immunostaining of rat ovary using antiserum to purified rat liver 11β-hydroxysteroid dehydrogenase, displaying marked staining of the oocyte (×400).

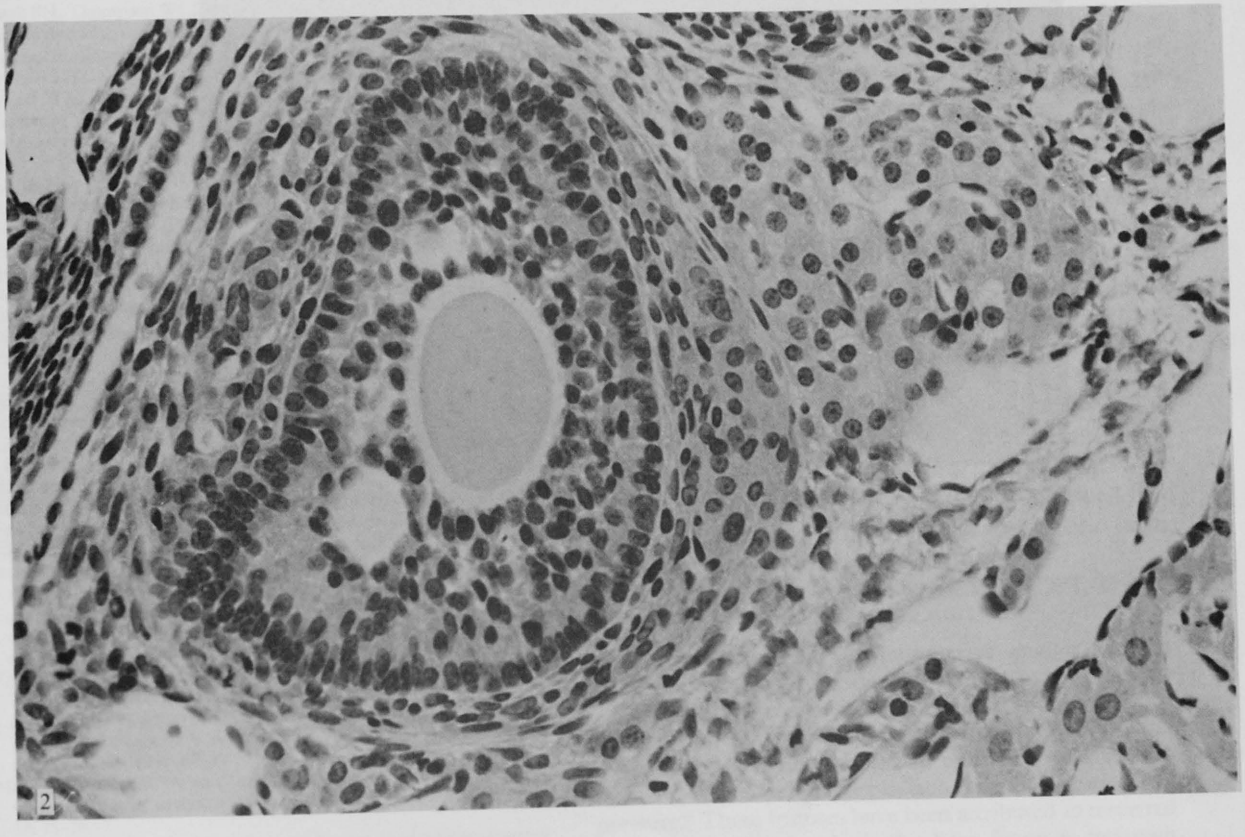
FIGURE 2. Control using preimmune serum, showing no immunostaining (×400).

Plate 2

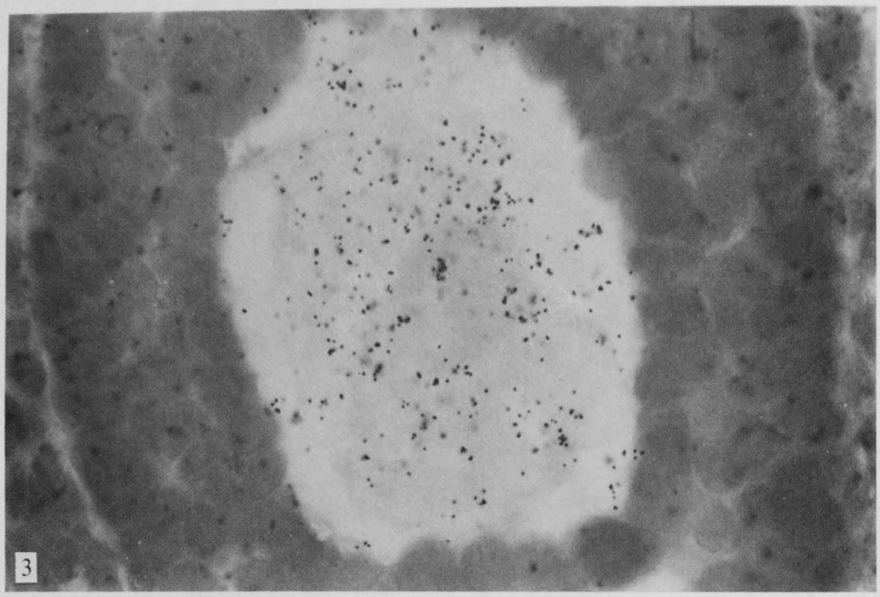
FIGURE 3. In-situ hybridization showing a concentration of silver grains over the oocyte indicating hybridization of the cRNA probe containing ^{35}S-labelled UTP to 11β-hydroxysteroid dehydrogenase mRNA (×600).

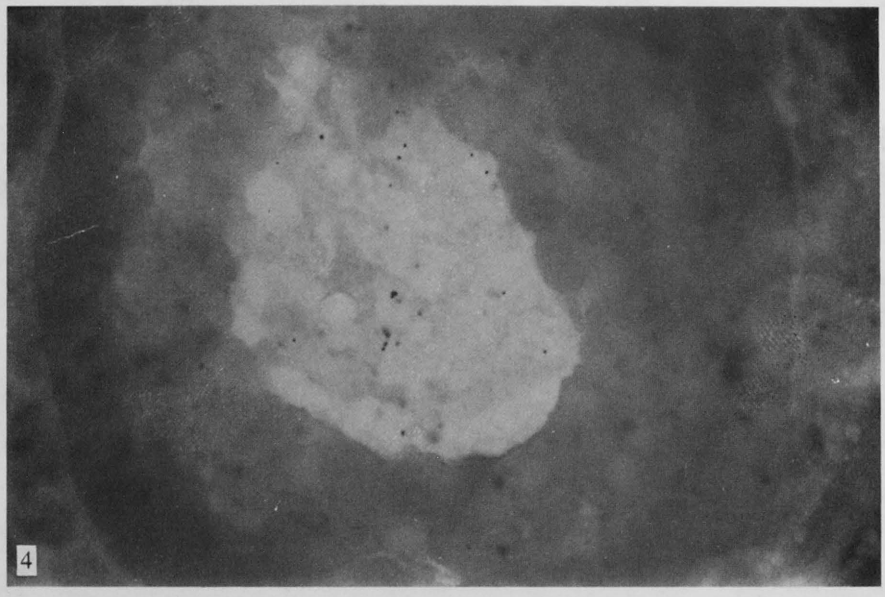
FIGURE 4. In-situ hybridization using 'sense' probe, showing no silver grain concentration (×600).





(Facing p. 58)


3


4

eded, and serum levels are almost always in the required nge, making control of these levels less important.

We thank Dr E. H. H. Wiltink for enthusiastic help during various stages of study; Teun Grooters for assistance in collecting data; Dr G. J. Weverling statistical advice; Dr Ir W. A. Dreschler and his staff for the audiograms; f J. Dankert, department of medical microbiology, for cooperation and vice; and all the residents and staff members who cooperated by warning us en aminoglycoside therapy was started in their patients.

REFERENCES

1. Kahlmeter G, Dahlager JI. Aminoglycoside toxicity: a review of clinical studies published between 1975 and 1982. *J Antimicrob Chemother* 1984; **13** (suppl A): 9–22.
2. Gilbert DN. Once-daily aminoglycoside therapy. *Antimicrob Agents Chemother* 1991; **35:** 399–405.
3. Mattie H, Craig WA, Pechère JC. Determinants of efficacy and toxicity of aminoglycosides. *J Antimicrob Chemother* 1989; **24:** 281–93.
4. Kapusnik JE, Hackbarth CJ, Chambers HF, Carpenter T, Sande ME. Single, large, daily dosing versus intermittent dosing of tobramycin for treating experimental pseudomonas pneumonia. *J Infect Dis* 1988; **158:** 7–12.
5. Vogelman B, Gudmundsson S, Turnidge J, Leggett J, Craig WA. In vivo postantibiotic effect in a thigh infection in neutropenic mice. *J Infect Dis* 1988; **157:** 287–98.
6. Vogelman BS, Craig WA. Postantibiotic effects. *J Antimicrob Chemother* 1985; **15** (suppl A): 37–46.
7. Van der Auwera P, Klastersky J. Serum bactericidal activity and postantibiotic effect in serum of patients with urinary tract infection receiving high-dose amikacin. *Antimicrob Agents Chemother* 1987; **31:** 1061–68.
8. Verpooten GA, Giuliano RA, Verbist L, Eestermans G, De Broe ME. Once-daily dosing decreases renal accumulation of gentamicin and netilmicin. *Clin Pharmacol Ther* 1989; **45:** 22–27.
9. De Broe ME, Verbist L, Verpooten GA. Influence of dosage schedule on renal cortical accumulation of amikacin and tobramycin in man. *J Antimicrob Chemother* 1991; **27** (suppl C): 41–47.
10. Powell SH, Thompson WL, Luthe MA, et al. Once-daily vs continuous aminoglycoside dosing: efficacy and toxicity in animal and clinical studies of gentamicin, netilmicin, and tobramycin. *J Infect Dis* 1983; **147:** 918–32.
11. Bennett WM, Plamp CE, Gilbert DN, Parker RA, Porter GA. The influence of dosage regimen on experimental gentamicin nephrotoxicity: dissociation of peak serum levels from renal failure. *J Infect Dis* 1979; **140:** 576–80.
12. Tran Ba Huy P, Bernard P, Schacht J. Kinetics of gentamicin uptake and release in the rat. Comparison of inner ear tissues and fluids with other organs. *J Clin Invest* 1986; **77:** 1492–500.
13. Proctor L, Petty B, Lietman P, Thakor R, Glackin R, Shimizu H. A study of potential vestibulotoxic effects of once daily versus thrice daily administration of tobramycin. *Laryngoscope* 1987; **97:** 1443–49.
14. Ter Braak EW, De Vries PJ, Bouter KP, et al. Once-daily dosing regimen for aminoglycoside plus β-lactam combination therapy of serious bacterial infections: comparative trial with netilmicin plus ceftriaxone. *Am J Med* 1990; **89:** 58–66.
15. De Vries PJ, Verkooyen RP, Leguit P, Verbrugh HA. Prospective randomized study of once-daily versus thrice-daily netilmicin regimens in patients with intraabdominal infections. *Eur J Clin Microbiol Infect Dis* 1990; **9:** 161–68.
16. Hollender LF, Bahnini J, De Manzini N, et al. A multicentric study of netilmicin once daily versus thrice daily in patients with appendicitis and other intra-abdominal infections. *J Antimicrob Chemother* 1989; **23:** 773–83.
17. Sturm AW. Netilmicin in the treatment of gram-negative bacteremia: single daily versus multiple daily dosage. *J Infect Dis* 1989; **159:** 931–37.
18. Tulkens PM. Pharmacokinetic and toxicological evaluation of a once-daily regimen versus conventional schedules of netilmicin and amikacin. *J Antimicrob Chemother* 1991; **27** (suppl C): 49–61.
19. Van der Auwera P, Meunier F, Ibrahim S, Kaufman L, Derde MP, Tulkens PM. Pharmacodynamic parameters and toxicity of netilmicin (6 milligrams/kilogram/day) given once daily or in three divided doses to cancer patients with urinary tract infection. *Antimicrob Agents Chemother* 1991; **35:** 640–47.
20. Maller R, Isaksson B, Nilsson L, Sörén L. A study of amikacin given once versus twice daily in serious infections. *J Antimicrob Chemother* 1988; **22:** 75–79.
21. Maller R, Ahrne H, Eilard T, Eriksson I, Lausen I and the Scandinavian Amikacin Once Daily Study Group. Efficacy and safety of amikacin in systemic infections when given as a single daily dose or in two divided doses. *J Antimicrob Chemother* 1991; **27** (suppl C): 121–28.
22. Knaus WA, Draper EA, Wagner DP, Zimmerman JE. APACHE II: a severity of disease classification system. *Crit Care Med* 1985; **13:** 818–29.
23. Cockcroft DW, Gault MH. Prediction of creatinine clearance from serum creatinine. *Nephron* 1976; **16:** 31–41.
24. Hull JH, Sarubbi FA. Gentamicin serum concentrations: pharmacokinetic predictions. *Ann Intern Med* 1976; **85:** 183–89.
25. Balows A, Hausler WJ Jr, Herrmann KL, Isenberg HD, Shadomy HJ, eds. Manual of clinical microbiology. 5th ed. Washington: American Society for Microbiology 1991.
26. Smith CR, Lipsky JJ, Laskin OL, et al. Double-blind comparison of the nephrotoxicity and auditory toxicity of gentamicin and tobramycin. *N Engl J Med* 1980; **302:** 1106–09.
27. Thomas DG, Gart JJ. A table of exact confidence limits for differences and ratios of two proportions and their odds ratios. *J Am Statist Assoc* 1977; **72:** 73–76.
28. Cheung R, Clark P, Nicholson PW, et al. Screening for aminoglycoside auditory toxicity in the old. *Br J Clin Pharmacol* 1990; **30:** 1–11.
29. Brummett RE, Fox KE. Aminoglycoside-induced hearing loss in humans. *Antimicrob Agents Chemother* 1989; **33:** 797–800.
30. Smith CR, Ambinder R, Lipsky JJ, et al. Cefotaxime compared with nafcillin plus tobramycin for serious bacterial infections. A randomized, double-blind trial. *Ann Intern Med* 1984; **101:** 469–77.
31. Eisenberg JM, Koffer H, Glick HA. What is the cost of nephrotoxicity associated with aminoglycosides? *Ann Intern Med* 1987; **107:** 900–09.

SHORT REPORTS

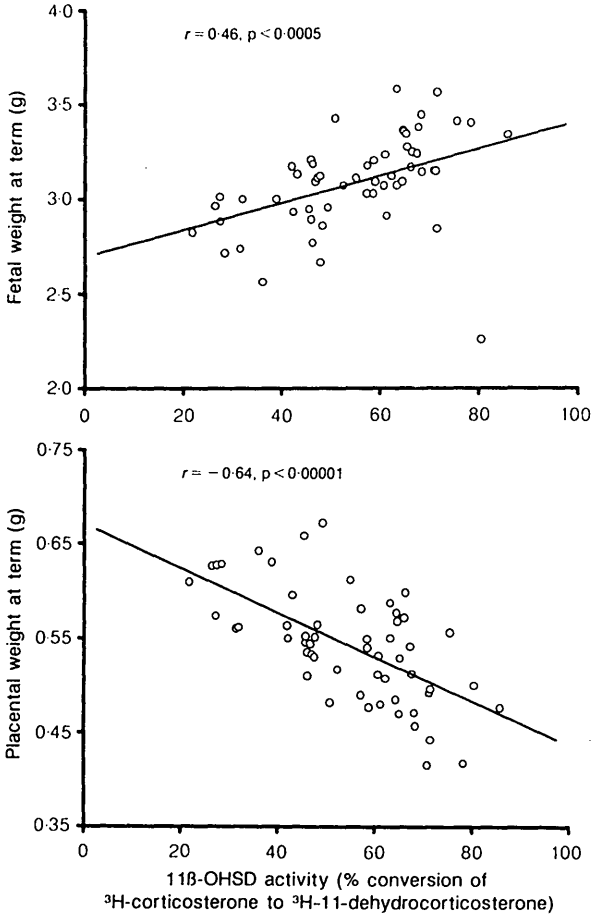
Glucocorticoid exposure in utero: new model for adult hypertension

RAFN BENEDIKTSSON ROBERT S. LINDSAY
JUNE NOBLE JONATHAN R. SECKL
CHRISTOPHER R. W. EDWARDS

Hypertension is strongly predicted by the combination of low birthweight and a large placenta. This association could be due to increased fetal exposure to maternal glucocorticoids. Fetal protection is normally effected by placental 11β-hydroxysteroid dehydrogenase (11β-OHSD), which converts physiological glucocorticoids to inactive products. We found that rat placental 11β-OHSD activity correlated positively with term fetal weight and negatively with placental weight. Offspring of rats treated during pregnancy with dexamethasone (which is not metabolised by 11β-OHSD) had lower birthweights and higher blood pressure when adult than did offspring of control rats. Increased fetal glucocorticoid exposure secondary to attenuated placental 11β-OHSD activity may link low birthweight and high placental weight with hypertension.

Lancet 1993; **341**: 339–41.

The importance of the intrauterine environment in relation to cardiovascular disease has been emphasised by epidemiological studies showing that low birthweight is a marker for ischaemic heart disease mortality[1] and hypertension.[2] Adults who had the unusual combination of low birthweight and a large placenta have the highest blood pressure.[2] These features have been attributed to maternal malnutrition, but they have also been found in rats with streptozotocin-induced diabetes mellitus,[3,4] which have high maternal glucocorticoid concentrations.[5] Furthermore, exogenous glucocorticoids retard fetal growth in human beings and animals.[6]



Correlations of term fetal weight and placental weight with placental 11β-OHSD activity.

11β-hydroxysteroid dehydrogenase (11β-OHSD) converts cortisol and corticosterone to inactive products (cortisone, 11-dehydrocorticosterone), and placental 11β-OHSD protects the fetus from exposure to the growth-inhibiting effects of maternal glucocortocoids;[7] thus fetal cortisol concentrations are much lower than those in the maternal circulation.[8] Since the effectiveness of this placental glucocorticoid barrier could vary, we examined 11β-OHSD activity in rat placentas at term, and investigated the effects of treatment of pregnant rats with the non-metabolised glucocorticoid dexamethasone on offspring birthweight and blood pressure.

Eight Wistar rats (240 g) were killed at term, and the placentas (n = 56) were weighed and homogenised in Krebs-Ringer bicarbonate buffer. Enzyme assay was carried out in duplicate incubations with 0·5 g/L total placental protein, 200 μmol/L NAD (co-substrate), and 12 nmol/L 1,2,6,7,³H-corticosterone in buffer (plus 0·2% bovine serum albumin and glucose) for 1 h at 37°C. Steroids were separated by thin-layer chromatography and enzyme activity expressed as percentage conversion of ³H-corticosterone to ³H-11-dehydrocorticosterone. Further groups of rats were treated with dexamethasone (100 μg/kg daily subcutaneously; n = 5) or vehicle (4% ethanol-saline, n = 6) throughout pregnancy. All treatment was stopped at birth and the pups were reared identically. When the offspring were 140–150 days old carotid cannulae were inserted under halothane anaesthesia and blood pressure was measured on 3 separate days at least 48 h after cannulation. Comparison was by Student's unpaired *t* test.

MEAN (SE) BLOOD PRESSURE (BP; mm Hg) IN OFFSPRING OF DEXAMETHASONE-TREATED AND CONTROL RATS

—	Treated	Control	p
Male offspring			
n	7	11	
Systolic BP	115 (4)	142 (4)	<0·05
Diastolic BP	122 (3)	115 (3)	0·09
Female offspring			
n	14	16	
Systolic BP	140 (2)	131 (3)	<0·05
Diastolic BP	103 (3)	102 (3)	0·82

There was a strong positive correlation between placental 11β-OHSD activity and fetal weight at term and an inverse correlation between placental 11β-OHSD and placental weight (figure). Both correlations remained significant on analysis of mean values for each litter (for mean birthweight, n = 8, $r = 0·96$, $p < 0·001$; for mean placental weight n = 8, $r = -0·66$, $p < 0·05$). Maternal weight gain was significantly lower in dexamethasone-treated than control rats (mean 47·3 [SE 2·4] *vs* 80·0 [3·9]g in controls, $p < 0·0001$) but there was no effect on litter size (9·5 [0·5] *vs* 9·6 [0·6]) or gestation length (22·5 [0·2] *vs* 22·0 [0·3] days). Birthweight was lower in dexamethasone-treated than control rats (4·75 [0·08] *vs* 5·62 [0·08] g, $p < 0·05$). Maternal dexamethasone treatment significantly increased systolic blood pressure in both male and female adult offspring (table).

Our hypothesis that relative glucocorticoid excess in utero might cause subsequent hypertension is supported by the findings that placental 11β-OHSD activity correlates directly with birthweight and inversely with placental weight. Fetuses with low birthweight and high placental weight (which in man are at highest risk of subsequent hypertension[2]) had the lowest 11β-OHSD activity and presumably therefore the greatest exposure to maternal glucocortocoids. Excess glucocorticoids inhibit fetal growth in rats and human beings.[6] The mechanism of disproportionate placental enlargement remains unclear; rather than being a direct glucocorticoid effect on the placenta it could be secondary (or compensatory) to a failure of fetal growth as observed in hypoxia and anaemia.[9]

The development of adult hypertension in rats exposed to excess glucocorticoid in utero, in doses not affecting fetal viability, also supports our hypothesis. Although our study did not address the in-utero mechanisms that brought about subsequent hypertension, direct actions of glucocorticoids on fetal tissue development or hormonal imprinting may be important. Indirect effects cannot be excluded, since offspring blood pressure is related to maternal blood pressure during pregnancy[2] and dexamethasone may raise maternal blood pressure.[10] These findings lead to a testable hypothesis to explain epidemiological data linking impaired human fetal growth and common disorders in later life, especially hypertension.

This study was supported by the Wellcome Trust (C. R. W. E. and J. S.) and the Scottish Hospitals Endowment Research Trust (C. R. W. E. and R. B.).

REFERENCES

1. Barker DJP, Osmond C, Winter PD, Margetts B. Weight in infancy and death from ischaemic heart disease. *Lancet* 1989; ii: 577–80.
2. Barker DJP, Bull AR, Osmond C, Simmonds SJ. Fetal and placental size and risk of hypertension in adult life. *BMJ* 1990; **301:** 259–62.
3. Robinson J, Canavan JP, El Haj AJ, Goldspink DF. Maternal diabetes in rats: I, effects on placental growth and protein turnover. *Diabetes* 1988; **37:** 1665–70.
4. Canavan JP, Goldspink DF. Maternal diabetes in rats: II, effects on fetal growth and protein turnover. *Diabetes* 1988; **37:** 1671–83.
5. Heller CL, Weisenberg LS, Orti E, De Nicola AF. Steps of glucocorticoid action in normal and diabetic rat placenta. *J Steroid Biochem* 1988; **31:** 119–23.
6. Reinisch JM, Simon NG, Karow WG, Gandelman R. Prenatal exposure

to prednisone in humans and animals retards intrauterine growth. *Science* 1978; **202**: 436–38.
7. Beitins IZ, Bayard F, Ances IG, Kowarski A, Migeon CJ. The metabolic clearance rate, blood production, interconversion and transplacental passage of cortisol and cortisone in pregnancy near term. *Pediatr Res* 1973; **7**: 509–19.
8. Edwards CRW, Benediktsson R, Lindsay RS, Seckl JR. Dysfunction of placental glucocorticoid barrier: link between fetal environment and adult disorders? *Lancet* 1993; **341**: 355–57.
9. Alexander G. Factors regulating the growth of the placenta: with comments on the relationship between placental weight and fetal weight. In: Naftolin F, ed. Abnormal fetal growth: biological bases and consequences. Berlin: Abakon Verlagsgesellschaft, 1978: 149–64.
10. Tonolo G, Fraser R, Connell JMC, Kenyon CJ. Chronic low-dose infusions of dexamethasone in rats: effects on blood pressure, body weight and plasma atrial natriuretic peptide. *J Hypertens* 1988; **6**: 25–31.

ADDRESS: **University of Edinburgh, Department of Medicine, Western General Hospital, Edinburgh EH4 2XU, UK** (R. Benediktsson, CandMed, R. S. Lindsay, MRCP, J. Noble, AIAT, J. R. Seckl, PhD, Prof C. R. W. Edwards, FRCP). Correspondence to Prof C. R. W. Edwards.

Abnormal growth regulation of vascular smooth muscle cells by heparin in patients with restenosis

PHILIP CHAN MAHENDRA PATEL
LAURA BETTERIDGE EUAN MUNRO
MICHAEL SCHACHTER JOHN WOLFE
PETER SEVER

Proliferation of vascular smooth muscle cells (VSMC) underlies myointimal hyperplasia, which can lead to restenosis after angioplasty and vascular surgery. We propose that some individuals have an intrinsic capacity for this exaggerated response to vascular injury, partly through decreased sensitivity to the physiological growth inhibitor heparin. We investigated the effect of heparin on VSMC from restenotic lesions and from apparently normal vessels of the same patients, and VSMC from control patients undergoing primary bypass procedures. Cells from patients with restenosis (both restenotic lesion and undiseased vein) showed much lower sensitivity to growth inhibition by heparin than the controls (median inhibition 8 [95% CI −2 to 25] *vs* 22 [15–44]%, $p<0{\cdot}001$); this finding suggests aberrant growth regulation in these cells.

Lancet 1993; **341**: 341–42.

Myointimal hyperplasia (MIH), a physiological response to vascular injury, occurs after every interventional vascular procedure. Proliferation of vascular smooth muscle cells (VSMC) forms the cellular basis of MIH and also underlies atherogenesis and vascular transplant rejection.[1] Vascular bypass surgery and balloon angioplasty are among the commonest procedures in western societies. In about 20% of vein grafts and 30–40% of coronary angioplasties MIH is progressive and associated with significant restenosis. The restenosis usually results in the recurrence of ischaemic symptoms or in tissue damage and is an important cause of treatment failure. It is not clear why there are differences in patient response after vascular procedures. Progressive MIH cannot be predicted by technical or haemodynamic factors, and systemic risk factors such as diabetes, hypertension, continued smoking, and hyperlipidaemia are not consistently associated with restenosis. Furthermore, some patients suffer repeated restenosis, which suggests variations in individual susceptibility to progressive MIH.

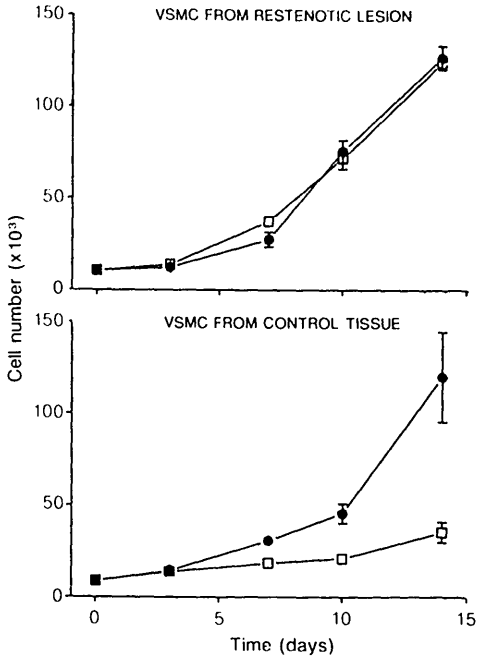


Fig 1—Representative VSMC growth curves (mean and SD cell counts) for 1 restenosis patient and 1 control with (□) and without (●) heparin.

The factors that regulate growth of VSMC remain unclear. After endothelial injury VSMC are subject to paracrine and autocrine regulation by growth factors from damaged endothelial cells, platelets, macrophages/monocytes, and VSMC.[1] We have tested the hypothesis that VSMC from patients who develop restenosis have intrinsically abnormal growth regulatory mechanisms.

We have grown VSMC cultures from 136 samples of vascular tissue.[2] The cell cultures used in this study were grown from 43 vascular tissue samples obtained from 39 unselected patients undergoing cardiac and peripheral vascular surgery between December, 1990, and April, 1992. Cultures were established by an explant method modified from Chamley-Campbell[2] and grown in Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum and antibiotics. VSMC cultures had the characteristic hill and valley morphology and stained positively with an antibody to α-actin, a marker for smooth muscle cells, and negatively for factor-VIII-related antigen. Cells were plated at the third passage at 10^4/mL and allowed to attach for 24 h. The 14 day growth assay was preceded by 72 h of growth arrest with 0·4% fetal calf serum. Growth was then restarted by addition of medium with 15% fetal calf serum, with or without unfractionated heparin 100 μg/mL. Cell counts on triplicate wells were done on days 0, 3, 7, 10, and 14 on a Coulter counter.

Vascular tissues for this study were classified as follows. Restenosis cells were derived from patients undergoing reoperation for graft stenosis (from the restenotic lesion in 8 and from apparently undiseased saphenous vein in 6). 6 samples of control arteries (2 internal mammary artery, 1 common femoral artery, 1 popliteal artery, and 2 iliac artery) and 29 samples of saphenous vein were obtained from organ donors ($n=2$) and patients undergoing primary cardiovascular surgery. All tissue samples included the full thickness of the vessel wall. Ideal control subjects would be patients who had undergone vascular surgery and not developed restenosis; however, such subjects are not available. We therefore chose to use unselected subjects undergoing primary bypass procedures, to reflect the patient population requiring vascular intervention. The use of vascular tissues discarded at surgery complies with the guidelines of our ethics committee.

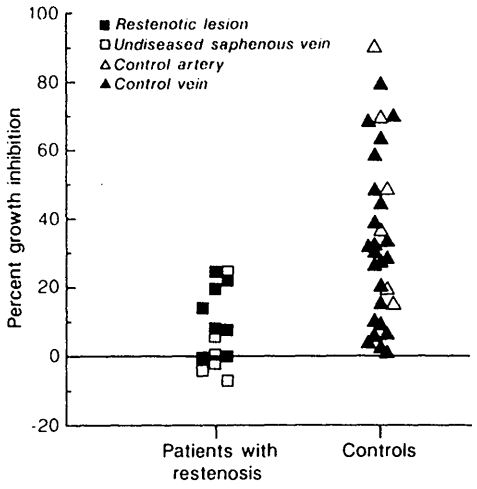


Fig 2—Summary of growth inhibition (means for individual cell strains) of VSMC by heparin.

$$\text{Inhibition} = 1 - \left(\frac{\text{cell no day 14 with heparin}}{\text{cell no day 14 without heparin}}\right) \times 100.$$

There was no significant difference in VSMC growth rate between those from restenotic lesions and those from control samples (mean increase during 14 days 812 [SE 151] *vs* 986 [102]%; population doubling time 2·84 [0·34] *vs* 2·47 [0·31] days). This finding contrasts with a report that VSMC from restenotic coronary lesions grow more rapidly than those from primary plaques.[3]

There was, however, a clear difference in sensitivity to heparin between control cells and cells from patients with restenosis, whether from the lesion or from undiseased saphenous vein (fig 1). Heparin inhibited the increase in the number of control cells at 14 days by a median of 28% (95% CI 15–44; n = 29), whereas the proliferation of cells from restenotic lesions and normal vessels from the patients with restenosis was inhibited by only 8% (–2 to 25; n = 14; $p < 0{\cdot}001$, Mann-Whitney test; fig 2).

Heparin was the first described inhibitor of VSMC growth both in vivo and in vitro.[4] Endogenous glycosaminoglycans similar to heparin, produced by endothelium and VSMC are involved in growth regulation of these cells. The heparin concentration used (100 μg/mL) is higher than concentrations normally attained in serum after therapeutic doses but similar to concentrations in the extracellular matrix around the VSMC of the vessel wall.[5] The effect of heparin on growth inhibition cannot involve its antithrombotic action, since these cells are grown in preclotted fetal calf serum.

The mechanism of heparin inhibition is unclear. Heparin-like substances may change the extracellular matrix, reducing the proliferative capacity of VSMC.[6] Heparin interacts with thrombospondin, a secreted glycoprotein involved in VSMC mitogenesis, and may reduce the production of tissue plasminogen activator and metalloproteinases, which are involved in cell migration and proliferation.[7] Furthermore, heparin inhibits protein kinase C and the expression of cellular proto-oncogenes that depends on this enzyme.[8]

It is unlikely that the reduced heparin sensitivity of VSMC from patients with restenosis could be due to residual effects of drug treatments, because the cells had been passaged at least twice. Heparin resistance in normal vascular tissue may indicate a propensity for the development of restenosis. The growth potential of VSMC from these patients may antedate vascular injury and therefore represent a risk factor at the cellular level for progressive MIH.

About 30–40% of patients undergoing primary bypass surgery, such as our controls, will develop restenosis; thus, any differences between restenosis and control VSMC would tend to be minimised in this study. The existence of persistently significant differences between groups indicates the robust nature of the findings. There was a wide range of heparin sensitivity among controls (fig 2); a prospective analysis would be required to correlate the sensitivity with the development of restenosis.

The implications of these findings are important. The search for pharmacological treatments to prevent stenosis is based on observations in animal models of vascular injury. We have defined an aberration in the growth regulation of human VSMC that is associated with restenosis. No corresponding findings in animals have been reported, although Resink et al[9] have shown that VSMC from spontaneously hypertensive rats have lower sensitivity to growth inhibition by heparin than VSMC from Wistar Kyoto rats. We propose that animal models are of dubious validity unless similarities to human tissue can be established. One report has even suggested that heparin treatment may promote restenosis.[10] The relative heparin resistance of human VSMC may explain why many pharmacological agents that inhibit MIH in animal models have failed to prevent human vascular restenosis.

This study was supported by the Institut de Récherches Internationales Servier, the Coronary Flow Trust, and Impra UK. E. M. is a British Heart Foundation junior research fellow and M. S. is a BHF senior research fellow. We thank Dr E. Kupek (St Mary's Hospital Medical School) for advice on statistics, and the surgeons and theatre staff at St Mary's Hospital for providing the vascular tissue for this study.

REFERENCES

1. Schwartz SM, Campbell GR, Campbell JH. Replication of smooth muscle cells in vascular disease. *Circ Res* 1986; **58**: 427–444.
2. Chan P, Munro E, Patel M, et al. Cellular biology of human intimal hyperplastic stenosis. *Eur J Vasc Surg* (in press).
3. Dartsch PC, Voisard R, Bauriedel G, Hofling B, Betz E. Growth characteristics and cytoskeletal organization of cultured smooth muscle cells from human primary stenosing and restenosing lesions. *Arteriosclerosis* 1990; **10**: 62–75.
4. Clowes AW, Karnovsky MJ. Suppression by heparin of smooth muscle cell proliferation in injured arteries. *Nature* 1977; **265**: 625–26.
5. Yla-Herttuala S, Sumuvuorhi H, Karkola K, Mottonen M, Nikkari T. Glycosaminoglycans in normal and atherosclerotic human coronary arteries. *Lab Invest* 1989; **54**: 402–07.
6. Snow AD, Bolender RP, Wight TN, Clowes AW. Heparin modulates the composition of the extracellular matrix domain surrounding arterial smooth muscle cells. *Am J Physiol* 1990; **137**: 313–30.
7. Au YPT, Kenagy RD, Clowes AW. Heparin selectively inhibits the transcription of tissue-type plasminogen activator in primate arterial smooth muscle cells during mitogenesis. *J Biol Chem* 1992; **267**: 3438–44.
8. Pukac LA, Ottlinger ME, Karnovsky MJ. Heparin suppresses specific second messenger pathways for protooncogene expression in rat vascular smooth muscle cells. *J Biol Chem* 1992; **267**: 3707–11.
9. Resink TJ, Scott-Burden T, Baur U, Burgin M, Buhler FR. Decreased susceptibility of culture smooth muscle cells from SHR rats to growth inhibition by heparin. *J Cell Physiol* 1989; **138**: 137–44.
10. Lehmann KG, Doria RJ, Feuer JM, Hall PX, Hoang DT. Paradoxical increase in restenosis rate with chronic heparin use: final results of a randomized trial. *J Am Coll Cardiol* 1991; **17**: 181A.

ADDRESSES: **Department of Clinical Pharmacology, St Mary's Hospital Medical School, Imperial College of Science, Technology and Medicine** (P. Chan, FRCS, M. Patel, PhD, L. Betteridge, BSc, E. Munro, FRCS, M. Schachter, MRCP, Prof P. Sever, FRCP); **and Department of Vascular Surgery, St Mary's Hospital, London, UK** (P. Chan, E. Munro, J. Wolfe, FRCS). Correspondence to Mr P. Chan, Department of Clinical Pharmacology, St Mary's Hospital Medical School, London W2 1NY, UK.

HYPOTHESIS

Dysfunction of placental glucocorticoid barrier: link between fetal environment and adult hypertension?

CHRISTOPHER R. W. EDWARDS RAFN BENEDIKTSSON
ROBERT S. LINDSAY JONATHAN R. SECKL

Low birthweight is associated with the subsequent development of common disorders of adult life, especially hypertension; maternal malnutrition has been suggested as the cause. We suggest an alternative aetiology—increased fetal exposure to maternal glucocorticoids. This hypothesis is supported by our findings that in rats decreased activity of the enzyme that acts as a placental barrier to maternal glucocorticoids (11β-hydroxysteroid dehydrogenase) is associated with low birthweight. Furthermore, increased exposure of the fetus to exogenous glucocorticoids leads to low birthweight and subsequent hypertension in the offspring. Glucocorticoids acting during critical periods of prenatal development may, like other steroid hormones, exert organisational effects or imprint patterns of response that persist throughout life. Thus, the lifetime risk of common disorders may be partly determined by the intrauterine environment.

Lancet 1993; **341**: 355–57.

Birthweight and disease

Epidemiological studies have strongly implicated intrauterine environmental factors in the risk of common disorders in later life. Low birthweight is strongly predictive of high blood pressure at all ages;[1-5] it is also associated with an increased risk of non-insulin-dependent diabetes mellitus and hyperinsulinaemia[6] and, presumably as a result of these, higher mortality from ischaemic heart disease.[7] The strongest predictor of adult hypertension is the unusual combination of low birthweight and a large placenta.[5]

Factors determining fetal and placental growth

Adult height is closely related to parental height, which suggests a strong genetic influence. Are uterine growth and birthweight similarly constrained? In crosses between large horses and small ponies, the size of the offspring and the placenta is directly proportional to maternal size and is not intermediate.[8] In human beings too, birthweight is strongly influenced by maternal height.[9] The correlation between birthweight and weight at 12 months is relatively poor, whereas weight at 12 months correlates closely with weight subsequently.[10] This finding suggests that in both animals and man there are local maternal factors, quite distinct from the maternal and paternal genes affecting the infant's ultimate size, which limit the growth of the fetus. Physical limitation of uterine space is not an important factor in rabbits,[9] although the evidence in man is less clear.[5] Whatever these maternal factors are, there could be catch-up growth after birth. It is interesting that adolescents with the highest blood pressures are those who grow fastest as children.[11]

There are clear geographical differences in the incidence of cardiovascular disease in the UK; the correlation between these variations and blood pressure,[12] suggests that there are important environmental determinants of blood pressure. Maternal smoking can affect fetal growth, but not placental weight.[14] Moreover, smoking is associated with proportional reductions in birthweight and length,[15] whereas in the Preston Study,[5] the growth-retardant effects in low-birthweight infants who developed hypertension in adulthood were not in proportion; for any birthweight, head circumference increased with placental weight, but body length decreased. The Preston investigators[5] suggested that there might be diversion of blood away from the trunk in favour of the brain, as occurs in animals made hypoxic in utero.[16,17] Hypoxia[18] and maternal anaemia[19] have also been associated with retarded fetal growth and disproportionate placental enlargement. In both cases the increase in placental size was judged to be compensatory. Alcohol excess during pregnancy can affect fetal growth and development. Chronic alcohol administration to pregnant rats leads to low fetal weight and high placental weight.[20] Unfortunately, smoking habits and alcohol intake were not recorded during the Preston study.[5]

It is not known whether any of the factors thought to cause hypertension, such as salt intake, affect blood pressure at an early age. Salt-sensitive rats fed a sodium-enriched diet from weaning to 16 weeks develop hypertension earlier than animals fed a control diet.[13] This effect seems to be irreversible—changing from high-salt to control diet after

ADDRESS: **University of Edinburgh Department of Medicine, Western General Hospital, Edinburgh EH4 2XU, UK** (Prof C. R. W. Edwards, FRCP, R. Benediktsson, Cand Med, R. S. Lindsay, MRCP, J. R. Seckl, PhD). Correspondence to Prof C. R. W. Edwards.

16 weeks has little effect on blood presure. There are no data on the effects of maternal sodium intake on fetal growth.

Maternal malnutrition has been suggested as a possible cause of impaired fetal growth.[5] Birthweight was reduced in the Netherlands during the severe privations of the wartime occupation during the 1940s.[21] The children subsequently showed a high risk of obesity.[22] Body weight and blood pressure are significantly related from childhood until middle age.[11] However, among sheep with singleton pregnancies, animals fed a restricted diet (resulting in only 0·5 kg weight gain during the last 60 days of pregnancy) and fully fed animals (gaining 17·7 kg during the same period) had lambs of similar birthweights.[8] Findings in cattle are similar.[23] Furthermore, in London during the war of 1939–45 (when some of the Preston cohort were in utero[5]) there was no evidence of an effect of the wartime diet on birthweight or the frequency of prematurity,[24] and wartime maternal supplementation with minerals and vitamins did not affect birthweight.[25] Moreover, in the Preston study[5] the relation between birthweight, placental weight, and adult blood pressure was found for each social class (at birth or 50 years later); thus it seems unlikely that variations in maternal nutrition due to social class affect birthweight. With the possible exception of extreme malnutrition, there is little evidence to link variations in maternal nutrition with intrauterine growth.

Prenatal exposure of the fetus to glucocorticoids retards intrauterine growth.[26,27] In our studies with low-dose dexamethasone, rat litter size was not affected but birthweight was significantly reduced.[28] Hydrocortisone can decrease or increase placental size in rats, perhaps depending on dose.[27] The association of low birthweight with a large placenta has been found in rats with streptozotocin-induced diabetes mellitus.[29,30] These animals have high glucocorticoid concentrations as well as hyperglycaemia.[31]

Glucocorticoids and the placenta

Most maternal cortisol crossing the human placenta is converted to biologically inactive cortisone.[32] Thus, under normal circumstances the fetus is protected from the growth-retarding effects of maternal glucocorticoids, which circulate at concentrations five to ten times higher than those in the fetus. Near term, the human fetal adrenal secretes 75% of circulating fetal cortisol, whereas fetal cortisone is mainly of maternal origin.[33] This protection is achieved by a placental enzyme, 11β-hydroxysteroid dehydrogenase (11β-OHSD), which rapidly converts physiological glucocorticoids to inactive products. The in-vitro activity of 11β-OHSD from the human placenta at term is oxidative (cortisol to cortisone), and there is negligible reduction of cortisone to cortisol.[34] Our studies in rats show that placental 11β-OHSD activity correlates positively with birthweight and negatively with placental weight.[28] Thus, fetuses with low birthweight and high placental weight (which in human studies are at highest risk of subsequent hypertension[5]) have the lowest 11β-OHSD activity and presumably, therefore, the greatest exposure to maternal glucocorticoids.

What happens if glucocorticoids do cross the placental barrier? Administration of low-dose dexamethasone to pregnant rats not only reduces birthweight but also leads to high blood pressure in young adult offspring.[28] The low, but supraphysiological, dose of dexamethasone we used did not affect fetal viability and attenuated the normal maternal weight gain only moderately, especially after correction for the lower fetal weight. Thus, we propose that abnormally low placental 11β-OHSD activity, by increasing fetal exposure to maternal glucocorticoids, leads to intrauterine growth retardation and high blood pressure in adulthood.

Regulators of placental 11β-OHSD

Low placental 11β-OHSD activity could be congenital or acquired. In rats, 11β-OHSD gene expression and enzyme activity are regulated in a tissue-specific manner by, for example, various hormones.[35,36] However, little is known about the regulation of placental 11β-OHSD. Oestrogen produced in the placenta from androgen precursors derived from the fetal adrenal stimulates enzyme-catalysed oxidation of cortisol to cortisone in baboons.[37] In-vitro studies of human placenta showed inhibition of 11β-OHSD by progesterone.[34] It is not known whether increased transplacental passage of maternal glucocorticoids secondary to 11β-OHSD inactivity (perhaps congenital) leads to suppression of fetal adrenal androgen production, causing further reduction in placental 11β-OHSD, but such excessive transfer could provide a cascade of deleterious changes in predisposed individuals. Another possibility is that maternal stress or changes in placental production or binding of corticotropin-releasing factor could lead to inhibition of placental 11β-OHSD by way of corticotropin, since excess endogenous or exogenous corticotropin causes inhibition of 11β-OHSD.[38]

The effects of attenuation of placental 11β-OHSD activity on fetal growth and offspring blood pressure are not yet clear. Conventional pharmacological methods may not be applicable, since inhibitors of 11β-OHSD are not completely selective, and we have found placental 11β-OHSD difficult to inhibit in vivo (unpublished). This finding may reflect the presence of a distinct isoform of 11β-OHSD in placenta with different biochemical characteristics from the liver enzyme (unpublished). Molecular cloning and expression of the placental enzyme and its manipulation in transgenic models may shed further light on its physological function.

Possible mechanisms linking in-utero glucocorticoid exposure with adult hypertension

Several studies have shown that there is perinatal plasticity of hormone receptors and enzymes. Csaba et al showed lasting amplification of insulin and vasopressin receptor responses after neonatal exposure to a single injection of the appropriate ligand,[39,40] an effect called hormonal imprinting. The permanent effects of steroid hormones given during the perinatal period are better established; brief neonatal exposure to sex steroids leads to permanent organisational changes in neuroanatomy, neuronal chemistry, and behaviour, which persist throughout adult life. Furthermore, there is strong evidence that steroids cause irreversible programming at birth of microsomal and soluble rat liver enzymes;[41,42] a single neonatal testosterone treatment permanently induces liver 5β-reductase, whereas 5α-reductase is suppressed throughout life. Glucocorticoids could similarly exert irreversible effects during a critical stage of development, leading to hypertension. How might this be mediated?

High concentrations of glucocorticoids in the fetus could affect the development of the fetal vasculature and its responses to pressor agents, since glucocorticoids modulate vascular tone by many mechanisms.[43] There is much evidence that human hypertension persists after removal of

the primary cause, possibly because of irreversible changes in vascular structure.[11] Glucocorticoids potently affect brain development[44] and another explanation is that glucocorticoid overexposure in utero leads to a failure of normal central control over cardiovascular growth and maturation, possibly by alteration of sympathetic efferents and responses to vasoactive stimuli. Furthermore, stressful events during the neonatal period change hypothalamic-pituitary-adrenal axis responses throughout life; these effects are mediated, at least partly, by permanent alterations of glucocorticoid receptor expression in specific regions of the nervous system.[45] We have found abnormal corticosteroid receptor gene expression in the brains of adult offspring of rats treated with dexamethasone during pregnancy (unpublished).

Indirect effects of changes in maternal metabolism during pregnancy or lactation or of high maternal blood pressure cannot be excluded. Offspring blood pressure is related to maternal blood pressure during pregnancy, and low-dose dexamethasone may raise blood pressure in rats.[46] Furthermore, the lasting effect on rats' blood pressure of neonatal exposure to excess sodium suggests that maternal sodium excess during pregnancy could lead to high blood pressure in the offspring. Thus the effect of glucocorticoids could be mediated by the mild sodium-retaining properties of dexamethasone in the fetus, the mother, or both. Physiological glucocorticoids, which have a higher affinity for the renal mineralocorticoid receptor than dexamethasone, may be more potent sodium retainers and this action might be increased by attenuated 11β-OHSD activity in the kidney, where the enzyme normally acts to protect the non-selective renal mineralocorticoid receptor from circulating cortisol or corticosterone.[47]

This work was supported by the Wellcome Trust (C. R. W. E. and J. S.) and by the Scottish Hospitals Endowment Research Trust (C. R. W. E. and R. B.).

REFERENCES

1. Barker DJP, Osmond C, Golding J, et al. Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease. *BMJ* 1989; **298:** 564–67.
2. Whincup PH, Cook DG, Shaper AG. Early influences on blood pressure: a study of children aged 5–7 years. *BMJ* 1989; **299:** 587–91.
3. Law CM, Barker DJP, Bull AR, et al. Maternal and fetal influences on blood pressure. *Arch Dis Child* 1991; **66:** 1291–95.
4. Seidman DS, Laor A, Gale R, et al. Birthweight, current body weight, and blood pressure in late adolescence. *BMJ* 1991; **302:** 1235–37.
5. Barker DJP, Bull AR, Osmond C, et al. Fetal and placental size and risk of hypertension in adult life. *BMJ* 1990; **301:** 259–62.
6. Hales CN, Barker DJP, Clark PMS, et al. Fetal and infant growth and impaired glucose tolerance at age 64. *BMJ* 1991; **303:** 1019–22.
7. Barker DJP, Osmond C, Winter PD, et al. Weight in infancy and death from ischaemic heart disease. *Lancet* 1989; ii: 577–80.
8. Hammond J. Physiological factors affecting birthweight. *Proc Nutr Soc* 1944; **2:** 8–12.
9. Butler N, Alberman ED. Second Report of the 1958 British Perinatal mortality survey. Edinburgh: Churchill Livingstone, 1969.
10. Tanner JM, Healy MJR, Lockhart RD, et al. Aberdeen Growth Study I: the prediction of adult body measurement from measurements taken each year from birth to 5 years. *Arch Dis Child* 1956; **31:** 372–81.
11. Lever AF, Harrap SB. Essential hypertension: a disorder of growth with origins in childhood? *J Hypertens* 1992; **10:** 101–20.
12. Shaper AG, Pocock SJ, Walker M, et al. British Regional Heart Study: cardiovascular risk factors in middle aged men in 24 towns. *BMJ* 1981; **283:** 179–86.
13. Hazon N, Henderson IW. Effects of altered dietary sodium intake on hormonal profiles in salt-sensitive hypertensive rats. *J Endocrinol* 1990; **127:** 243–48.
14. Spira A, Spira N, Goujrd J, et al. Smoking during pregnancy and placental weight: a multi-variant analysis of 3759 cases. *J Perinat Med* 1975; **3:** 237–41.
15. Miller HC, Hassanein K. Maternal smoking and fetal growth of full-term infants. *Pediatr* 1974; **8:** 960.
16. Campbell AGM, Dawes GS, Fishman AP, et al. Regional redistribution of blood flow in the mature foetal lamb. *Circ Res* 1967; **21:** 229–35.
17. Rudolph AM. The foetal circulation and its response to stress. *J Dev Physiol* 1984; **6:** 11–19.
18. Kroger H, Arias-Stella J. The placenta and the newborn infant at high altitudes. *Am J Obstet Gynecol* 1970; **106:** 586–91.
19. Godfrey KM, Redman CWG, Barker DJP, et al. The effect of maternal anaemia and iron deficiency on the ratio of fetal weight to placental weight. *Br J Obstet Gynecol* 1991; **98:** 886–91.
20. Sanchis R, Sancho TM, Guerri C. The effects of chronic alcohol consumption on pregnant rats and their offspring. *Alcohol* 1986; **21:** 295–305.
21. Stein Z, Susser M, Saenger G, et al. Famine and human development. In: The Dutch Hunger Winter of 1944–45. New York: Oxford University Press, 1975: 119–48.
22. Ravelli GP, Stein ZA Susser MV. Obesity in young men after famine exposure in utero and early infancy. *N Engl J Med* 1976; **295:** 349–53.
23. Eckles CH, Res Bull Min of Agriculture Exp Station 1919; 35.
24. Huggett A, St G. Diet in pregnancy. *Proc Nutr Soc* 1944; **2:** 20–27.
25. People's League of Health. *Lancet* 1942; **243:** 10.
26. Reinisch JM, Simon NG, Karow WG, et al. Prenatal exposure to prednisone in humans and animals retards intra-uterine growth. *Science* 1978; **202:** 436–38.
27. Gunberg DL. Some effects of exogenous hydrocortisone on pregnancy in the rat. *Anat Rec* 1957; **129:** 133–53.
28. Benediktsson R, Lindsay RS, Noble J, et al. Glucocorticoid exposure in utero: new model for adult hypertension. *Lancet* 1993; **341:** 339–41.
29. Robinson J, Canavan JP, El Haj AJ, et al. Maternal diabetes in rats I: effects on placental growth and protein turnover. *Diabetes* 1988; **37:** 1665–70.
30. Canavan JP, Goldspink DF. Maternal diabetes in rats II: effects on fetal growth and protein turnover. *Diabetes* 1988; **37:** 1671–83.
31. Heller CL, Weisenberg LS, Orti E, et al. Steps of glucocorticoid action in normal and diabetic rat. *J Steroid Biochem* 1988; **31:** 119–23.
32. Murphy BEP, Clark SJ, Donald IR, et al. Conversion of maternal cortisol to cortisone during placental transfer to the human fetus. *Am J Obstet Gynecol* 1974; **118:** 538–41.
33. Beitens IZ, Bayard F, Ances IG, et al. The metabolic clearance rate, blood production, interconversion and transplacental passage of cortisol and cortisone in pregnancy near term. *Pediatr Res* 1973; **7:** 509–19.
34. Lopez Bernal A, Flint APF, Anderson ABM, et al. 11β-hydroxysteroid dehydrogenase activity (E.C.1.1.1.146) in human placenta and decidua. *J Steroid Biochem* 1980; **13:** 1081–87.
35. Moisan M-P, Edwards CRW, Seckl JR. Ontogeny of 11β-hydroxysteroid dehydrogenase bioactivity and messenger RNA expression in rat brain and kidney. *Endocrinology* 1992; **130:** 400–04.
36. Monder C, Shackleton CHL. 11β-hydroxysteroid dehydrogenase: fact or fancy? *Steroids* 1984; **44:** 383–417.
37. Baggia S, Albrecht ED, Pepe GJ. Regulation of 11β-hydroxysteroid dehydrogenase activity in baboon placenta by oestrogen. *Endocrinology* 1990; **126:** 2742–48.
38. Walker BR, Campbell JC, Fraser R, et al. ACTH inhibits 11β-hydroxysteroid dehydrogenase in man: multiple actiologies of mineralocorticoid excess in ectopic ACTH syndrome. *Clin Endocrinol* (in press).
39. Csaba G, Dobozy O, Lazary G, et al. Possiblity of long-lasting amplification of insulin receptors by a single treatment at new born age. *Acta Phys Acat Scient Hung* 1979; **53:** 487–92.
40. Csaba G, Ronai A, Laszlo V, et al. Amplification of hormone receptors by a neonatal oxytocin and vasopressin treatment. *Horm Metab Res* 1980; **12:** 28–31.
41. Gustafsson GA, Stenberg A. Irreversible androgenic programming at birth of microsomal and soluble rat liver enzymes active on 4-androstene-3,17-dione and 5-alpha, androstane-3-alpha,17-beta-diol. *J Biol Chem* 1974; **249:** 711–18.
42. Gustafsson GA, Stenberg A. Neonatal programming of androgen responsiveness of liver of adult rats. *J Biol Chem* 1974; **249:** 719–23.
43. Walker BR, Williams BC. Corticosteroids and vascular tone: mapping the messenger maze. *Clin Sci* 1992; **82:** 597–605.
44. Mayer JS. Biochemical effects of corticosteroid on neural tissues. *Physiol Dev* 1985; **65:** 1020.
45. Meaney MJ, Aitken DH, Van Berkel C, et al. Effect of neonatal handling on age-related impairments associated with the hippocampus. *Science* 1988; **239:** 766–68.
46. Tonolo G, Fraser R, Connell JMC, et al. Chronic low-dose infusions of dexamethasone in rats: effect on blood pressure, body weight and plasma atrial natriuretic peptide. *J Hypertens* 1988; **6:** 25–31.
47. Edwards CRW, Stewart PM, Burt D, et al. Localisation of 11β-hydroxysteroid dehyrogenase-tissue specific protector of the mineralocorticoid receptor. *Lancet* 1988; ii: 986–89.

J. Steroid Biochem. Molec. Biol. Vol. 45, No. 1-3, pp. 1-5, 1993
Printed in Great Britain.

0960-0760/93 $6.00 + 0.00

CONGENITAL AND ACQUIRED SYNDROMES OF APPARENT MINERALOCORTICOID EXCESS

C. R. W. EDWARDS,* B. R. WALKER, R. BENEDIKTSSON and J. R. SECKL
Department of Medicine, Western General Hospital, Edinburgh EH4 2XU, Scotland

Summary—The enzyme 11β-hydroxysteroid dehydrogenase (11β-OHSD) interconverts cortisol and cortisone. Congenital deficiency of the renal isoform of the enzyme results in hypertension, hypokalemia and suppression of the renin–angiotensin–aldosterone system—the apparent mineralocorticoid excess syndrome (AME). In these patients cortisol acts as a potent mineralocorticoid. Suppression of plasma cortisol with dexamethasone results in natriuresis, potassium retention and reduction in blood pressure. Ingestion of excess liquorice or taking carbenoxolone produces an acquired form of AME. The active component of liquorice is glycyrrhetinic acid (GE) and carbenoxolone is the hemisuccinate derivative. Both GE and carbenoxolone are potent inhibitors of 11β-OHSD. *In vitro* studies have shown that 11β-OHSD is present in aldosterone-selective tissues and acts as an autocrine mechanism which prevents cortisol from gaining access to the non-specific mineralocorticoid receptor (MR). Congenital or acquired absence of this enzyme allows cortisol to bind to MR resulting in AME. 11β-OHSD also appears to be important in controlling cortisol access to glucocorticoid receptors. Variable placental 11β-OHSD may alter foetal exposure to maternal cortisol and affect growth as indicated by the correlation between foetal weight and placental 11β-OHSD. Thus the tissue-specific distribution, ontogeny and modulation of this enzyme allows it to dictate glucocorticoid effects in addition to its key role in ensuring the specificity of the MR.

INTRODUCTION

The apparent mineralocorticoid excess syndrome (AME) can be classified on the basis of whether it is congenital or acquired. The congenital syndrome has been further subdivided into types 1 and 2.

CONGENITAL AME

This was first described by Maria New and Stanley Ulick *et al.* [1, 2]. The syndrome's rather unusual name stemmed from the fact that the child described had all the biochemical features of gross mineralocorticoid excess with hypokalemia, hypertension and suppression of the renin–angiotensin system but no evidence of excess mineralocorticoid secretion could be found. The patient did not have Liddle's syndrome as judged by the failure to respond to inhibitors of renal tubular ionic transport. The key unexplained finding was the failure of conversion of cortisol to cortisone, as judged either by the very high ratio of cortisol to cortisone metabolites or the failure to metabolize [11α-^{3}H]cortisol to cortisone and [^{3}H]H_2O. Since then over 20 children and 1 adult with this syndrome have been reported [see 3 for review]. An even rarer syndrome has been described by Ulick *et al.* [4, 5] in which the clinical and basic biochemical features are identical but the ratio of urinary cortisol and cortisone metabolites is normal. This has been called the type 2 variant. There appears to be defective A ring reduction in these patients. It is not clear whether there is low 11β-hydroxysteroid dehydrogenase (11β-OHSD) activity. One possibility is that this is the case but it is not apparent in the ratio of urinary metabolites because it is masked by both the dehydrogenase and the reductase components of the enzyme being abnormal. In the type 1 syndrome reductase activity is normal as indicated by the normal conversion of cortisone to cortisol.

What was unclear from the initial studies was how a deficiency of 11β-OHSD resulted in the clinical picture of mineralocorticoid excess. New's group suggested that cortisol was acting as a mineralocorticoid in these patients [6]. Others demonstrated a beneficial effect of dexamethasone. We were able to study this in detail in the first adult patient to be described [7, 8].

Proceedings of the Symposium: Adrenal Steroids: Current Views and New Frontiers, Heidelberg, Germany, 4–6 June 1992.
*To whom correspondence should be addressed.

We showed that giving hydrocortisone 10 mg per day for 4 days resulted in marked sodium retention and kaliuresis. Conversely, suppression of cortisol production with dexamethasone produced natriuresis and potassium retention. Hypertension was improved but not normalized by dexamethasone. We suggested that defective renal inactivation of cortisol resulted in intrarenal cortisol excess which then gained access to the mineralocorticoid receptors (MR) in the cortical collecting tubule. We coined the term Cushing's disease of the kidney to graphically describe this [7]. To test this hypothesis we needed a model system in which we could observe the effects of inhibition of 11β-OHSD on the renal handling and effects of cortisol.

ACQUIRED AME

The effects of liquorice in producing a clinical picture which mimicked primary aldosteronism have been known for many years. This was thought to be a direct effect of the active component of liquorice (glycyrrhetinic acid, GE) on renal MR [9 for review]. However, early work had indicated that liquorice did not produce sodium retention in adrenalectomized subjects or patients with Addison's disease unless they were on hydrocortisone or cortisone replacement therapy. Others had shown that dexamethasone produced a natriuresis in patients taking GE [10] and that liquorice increased urinary free cortisol without altering plasma cortisol [11]. All this suggested to us that liquorice might act not directly but by inhibition of 11β-OHSD to produce an acquired form of AME. Our subsequent studies both *in vivo* in normal subjects and *in vitro* confirmed that GE is a potent inhibitor of 11β-OHSD [9, 12]. More recently Biglieri *et al.* [13] have confirmed inhibition of cortisol dehydrogenation in a liquorice addict.

Carbenoxolone, the hemisuccinate derivative of GE, also produces a mineralocorticoid excess syndrome but does not have the same kaliuretic effect as GE. Our studies have shown that it is an inhibitor of 11β-OHSD as judged by the failure of metabolism of [11α-^{3}H]cortisol but not when assessed by the ratio of urinary cortisol and cortisone metabolites [14]. Administration of cortisone to normal subjects taking carbenoxolone resulted in reduced plasma cortisol levels as compared to control indicating that carbenoxolone impaired reductase activity in addition to its effect on dehydrogenation. This raises the possibility that carbenoxolone therapy may be a model for the type 2 form of congenital AME.

ROLE OF 11β-OHSD IN PROTECTION OF MR

Studies by Funder's group [15, 16] demonstrated that the purified MR was non-selective and bound aldosterone and cortisol with equal affinity. Similar results were obtained by Arriza *et al.* [17] after cloning and expressing the human MR. Given that the circulating free plasma cortisol levels are approx. 100 times higher than those of aldosterone this indicated that there had to be some mechanism other than the structure of the receptor which defined its specificity. We suggested that 11β-OHSD was this mechanism and that in aldosterone-selective tissues the enzyme protected the MR from seeing cortisol by converting it to inactive cortisone [18]. Thus either congenital absence of the 11β-dehydrogenase or its inhibition by liquorice allowed cortisol to access the receptor and act as a mineralocorticoid. These results were confirmed by Funder *et al.* [19]. An essential part of this mechanism is that aldosterone is not a substrate for the enzyme. This would suggest that aldosterone is not present in the free 11β-hydroxyl form but as either the 11–18 hemiacetal or the 11–18–20 species. Studies using NMR have confirmed this with the 11–18 bridge form being the major one at physiological pH [20].

Further studies using either isolated tubules or cortical collecting duct cells *in vitro* have shown that 11β-OHSD is present in the same cells as the MR indicating that this is an autocrine system [21, 22]. Immunohistochemistry using an antiserum raised against purified hepatic 11β-OHSD, however, failed to show that the enzyme was in the distal nephron but was in the proximal tubule [18, 23]. It is now clear that the kidney contains at least two isoforms of the 11β-dehydrogenase and that the distal enzyme is NAD rather than NADP dependent [24, 25].

RELATIONSHIP OF 11β-OHSD TO GLUCOCORTICOID RECEPTORS (GR)

The distribution of 11β-OHSD in tissues not normally regarded as mineralocorticoid target tissues suggests that it may be important in controlling access of cortisol to GR in addition

to its MR protective role. Thus high concentrations of 11β-OHSD are found in the testis, the liver, cerebellum and lung. To demonstrate this possible role for the enzyme we used a classic assay of glucocorticoid activity, the cutaneous vasoconstrictor test [26]. Topical hydrocortisone applied to the forearm had very low vasoconstrictor activity as judged by the lack of blanching after overnight application. However, when both hydrocortisone and GE were applied together the glucocorticoid effect was significantly enhanced.

OTHER CONDITIONS IN WHICH DEFECTIVE 11β-OHSD MAY PLAY A ROLE

Congenital 11β-OHSD deficiency is very rare and liquorice abuse uncommon. However, there is evidence that there is a failure of conversion of cortisol to cortisone in other more common situations. These include chronic renal failure [27, 28], hypothyroidism [29], essential hypertension [30] and the ectopic ACTH syndrome [31, 32]. In a study of patients with essential hypertension we found that about one-third had impaired 11β-OHSD activity assessed by the prolonged half-life of [11α-^{3}H]cortisol [30]. However, despite this there was no evidence of activation of renal MR (no significant difference in plasma potassium, plasma renin activity, plasma aldosterone or urinary free cortisol). This raised the possibility that the site of abnormal metabolism might be in tissues such as the vasculature rather than the kidney [33].

Recent epidemiological studies by Barker *et al.* [34] in Southampton have demonstrated that low birth weight is associated with an increased risk for developing adult cardiovascular disease. They have shown a correlation of birth weight or length with ischaemic heart disease mortality [34], non-insulin dependent diabetes mellitus and hyperinsulinaemia [35], and hypertension [36, 37]. All these data pointed to the importance of the *in utero* environment in determining long-term cardiovascular risk. In the Preston study in which adults aged 50 were studied it was shown that those with the highest blood pressures were those who had been small babies with large placentas [37].

The mechanisms underlying these relationships have been obscure. It has been suggested that they might relate to maternal malnutrition but little evidence for this has been produced. It is also unclear how this could relate to the well-known genetic predisposition to the associated cardiovascular and metabolic conditions. Using an animal model we tested the hypothesis that variations in the *in utero* exposure to glucocorticoid might be the explanation.

11β-OHSD is present in high concentrations in the placenta [38] and has been thought to play a key role in the protection of the foetus from exposure to maternal glucocorticoids [39, 40]. We thought that this protection might not be absolute and that a relative deficiency of the placental inactivation mechanism might result in increased foetal glucocorticoid exposure with consequent reduction in growth. To test this we looked at the relationship between placental 11β-OHSD activity, birth weight and placental weight. In term rat placenta we found that enzyme activity was strongly positively correlated ($r = 0.46$, $P < 0.0005$) with foetal weight and negatively correlated with placental weight ($r = 0.63$, $P < 0.00001$) [41]. These results support our hypothesis. Further studies are required to show how *in utero* glucocorticoid excess might lead to hypertension in the adult. One attractive possibility is that there is hormonal imprinting. This has been demonstrated for several hormones such as vasopressin and insulin, and for the metabolism of steroids [42]. Hypokalemic alkalosis is a key feature of the ectopic ACTH syndrome in contrast to other forms of Cushing's syndrome. Ulick *et al.* [31] have suggested on the basis of studies in 2 patients that this might be due to overload of the cortisol inactivation mechanism. Our own results in a series of 26 patients with Cushing's syndrome (9 with ectopic ACTH secretion) indicate that the hypokalemic alkalosis results from a combination of mechanisms including cortisol overload, deoxycorticosterone and corticosterone excess and decreased inactivation of cortisol and corticosterone by the kidney [32].

REFERENCES

1. New M. I., Levine L. S., Biglieri E. G., Pariera J. and Ulick S.: Evidence for an unidentified steroid in a child with apparent mineralocorticoid hypertension. *J. Clin. Endocr. Metab.* **44** (1977) 924–933.
2. Ulick S., Levine L. S., Gunczler P., Zanconato G., Ramirez L. C., Rauh W., Rosler A., Bradlow H. L. and New M. I.: A syndrome of apparent mineralocorticoid excess associated with defects in the peripheral metabolism of cortisol. *J. Clin. Endocr. Metab.* **49** (1979) 757–764.
3. Walker B. R. and Edwards C. R. W.: 11β-Hydroxysteroid dehydrogenase and enzyme-mediated receptor

protection: life after liquorice? *Clin. Endocr.* **35** (1991) 281–289.
4. Ulick S., Chan C. K., Rao K. N., Edassery J. and Mantero F.: A new form of the syndrome of apparent mineralocorticoid excess. *J. Steroid Biochem.* **32** (1989) 209–212.
5. Ulick S., Tedde R. and Mantero F.: Pathogenesis of the type 2 variant of the syndrome of apparent mineralocorticoid excess. *J. Clin. Endocr. Metab.* **70** (1990) 200–206.
6. Oberfield S. E., Levine L. S., Carey R. M., Greig F., Ulick S. and New M. I.: Metabolic and blood pressure responses to hydrocortisone in the syndrome of apparent mineralocorticoid excess. *J. Clin. Endocr. Metab.* **56** (1983) 332–339.
7. Edwards C. R. W., Stewart P. M., Nairn I. M., Grieve J. and Shackleton C. H. L.: Cushing's disease of the kidney. *J. Endocr.* **104** (1985) 53. (Abstr.)
8. Stewart P. M., Corrie J. E. T., Shackleton C. H. L. and Edwards C. R. W.: Syndrome of apparent mineralocorticoid excess: a defect in the cortisol–cortisone shuttle. *J. Clin. Invest.* **82** (1988) 340–349.
9. Stewart P. M., Valentino R., Wallace A. M., Burt D., Shackleton C. H. L. and Edwards C. R. W.: Mineralocorticoid activity of liquorice: 11beta-hydroxysteroid dehydrogenase deficiency comes of age. *Lancet* **ii** (1987) 821–824.
10. Hoefnagels W. H. L. and Kloppenborg P. W. C.: Antimineralocorticoid effects of dexamethasone in subjects treated with glycyrrhetinic acid. *J. Hypert.* **1** (Suppl. 2) (1983) 313–315.
11. Epstein M. T., Espiner E. A., Donald R. A., Hughes H., Cowles R. J. and Lun S.: Liquorice raises urinary cortisol in man. *J. Clin. Endocr. Metab.* **47** (1978) 397–400.
12. Monder C., Stewart P. M., Lakshmi V., Valentino R., Burt D. and Edwards C. R. W.: Licorice inhibits corticosteroid 11β-dehydrogenase of rat kidney and liver: *in vivo* and *in vitro* studies. *Endocrinology* **125** (1989) 1046–1053.
13. Farese R. V., Biglieri E. G., Shackleton C. H. L. and Gomez-Fontes R.: Licorice-induced hypermineralocorticoidism. *New Engl. J. Med.* **325** (1991) 1223–1227.
14. Stewart P. M., Wallace A. M., Atherden S. M., Shearing C. H. and Edwards C. R. W.: Mineralocorticoid activity of carbenoxolone: contrasting effects of carbenoxolone and liquorice on 11β-hydroxysteroid dehydrogenase activity in man. *Clin. Sci.* **78** (1990) 49–54.
15. Krozowski Z. S. and Funder J. W.: Renal mineralocorticoid receptors and hippocampal corticosterone-binding species have identical intrinsic steroid specificity. *Proc. Natn. Acad. Sci. U.S.A* **80** (1983) 6056–6060.
16. Sheppard K and Funder J. W.: Mineralocorticoid specificity of renal type 1 receptors; *in vivo* binding studies. *Am. J. Physiol.* **252** (1987) E224–E229.
17. Arriza J. L., Weinberger C. and Cerelli G.: Cloning of human mineralocorticoid receptor complementary DNA; structural and functional kinship with the glucocorticoid receptor. *Science* **237** (1987) 268–275.
18. Edwards C. R. W., Stewart P. M., Burt D., Brett L., McIntyre M. A., Sutanto W. S., DeKloet E. R. and Monder C.: Localisation of 11beta-hydroxysteroid dehydrogenase—tissue specific protector of the mineralocorticoid receptor. *Lancet* **ii** (1988) 986–989.
19. Funder J. W., Pearce P. T., Smith R. and Smith A. I.: Mineralocorticoid action: target tissue specificity is enzyme, not receptor, mediated. *Science* **242** (1988) 583–585.
20. Edwards C. R. W. and Hayman A.: Enzyme protection of the mineralocorticoid receptor: Evidence in favour of the hemi-acetal structure of aldosterone. In *Aldosterone: Fundamental Aspects* (Edited by J. P. Bonvalet, N. Farman, M. Lombes and M. E. Rafestin-Oblin). Colloque INSERM/John Libbey Eurotext Ltd, Montrouge, **215** (1991) 67–76.
21. Fejes-Toth N. A., Watlington C. O. and Fejes-Toth G.: 11β-Hydroxysteroid dehydrogenase activity in the renal target cells of aldosterone. *Endocrinology* **129** (1991) 17–21.
22. Bonvalet J.-P., Doignon I., Blot-Chabaud M., Pradelles P. and Farman N.: Distribution of 11β-hydroxysteroid dehydrogenase along the rabbit nephron. *J. Clin. Invest.* **86** (1990) 832–837.
23. Rundle S. E., Funder J. W., Lakshmi V. and Monder C.: The intrarenal localization of mineralocorticoid receptors and 11β-dehydrogenase: immunocytochemical studies. *Endocrinology* **125** (1989) 1700–1704.
24. Walker B. R., Campbell J. C., Williams B. C. and Edwards C. R. W.: Tissue-specific distribution of the NAD + -dependent isoform of 11β-hydroxysteroid dehydrogenase. *Endocrinology* **131** (1992) 970–972.
25. Provencher P. H., Mercer W. R., Funder J. W. and Krozowski Z. S.: Identification and characterisation of an NAD-dependent 11β-HSD in pig kidney. *74th Meet. Am. Endocrine Soc.* San Antonio, TX (1992) Abstr. 305.
26. Teelucksingh S., Mackie A. D. R., Burt D., McIntyre M. A., Brett L. and Edwards C. R. W.: Potentiation of hydrocortisone activity in skin by glycyrrhetinic acid. *Lancet* **335** (1990) 1060–1063.
27. Whitworth J. A., Stewart P. M., Burt D., Atherden S. M. and Edwards C. R. W.: The kidney is the major site of cortisone production in man. *Clin. Endocr.* **31** (1989) 355–361.
28. Vierhapper H., Derfler K., Nowotny P., Hollenstein-U. and Waldhausl W.: Impaired conversion of cortisol to cortisone in chronic renal insufficiency—a cause of hypertension or an epiphenomenon? *Acta Endocr. (Copenh.)* **125** (1991) 160–164.
29. Richards A. M., Nicholls M. G., Espiner E. A., Ikram H., Turner J. G. and Brownlie B. E. W.: Hypertension in hypothyroidism: arterial pressure and hormone relationships. *Clin. Exp. Hypert.* **7** (1985) 1499–1514.
30. Walker B. R., Shackleton C. H. L. and Edwards C. R. W.: 11Beta-hydroxysteroid dehydrogenase deficiency in essential hypertension. *Proc. Endocrine Soc.* **73** (1991) 1378. (Abstr.)
31. Ulick S., Wang J. Z., Blumenfeld J. D. and Pickering T. G.: Cortisol inactivation overload: A mechanism of mineralocorticoid hypertension in the ectopic adrenocorticotropin syndrome. *J. Clin. Endocr. Metab.* **74** (1992) 963–967.
32. Walker B. R., Campbell J. C., Fraser R., Stewart P. M. and Edwards C. R. W.: ACTH inhibits 11β-hydroxysteroid dehydrogenase in man: Multiple aetiologies of mineralocorticoid excess in ectopic ACTH syndrome. *Clin. Endocr.* **27** (1992) 483–492.
33. Walker B. R., Yau J. L., Brett L. P., Seckl J. R., Monder C., Williams B. C. and Edwards C. R. W.: 11β-Hydroxysteroid dehydrogenase in vascular smooth muscle and heart: implications for cardiovascular responses to glucocorticoids. *Endocrinology* **129** (1991) 3305–3312.
34. Barker D. J. P., Osmond C., Winter P. D. and Margetts B.: Weight in infancy and death from ischaemic heart disease. *Lancet* **ii** (1989) 557–580.
35. Hales C. N., Barker D. J. P., Clark P. M. S., Cox L. J., Fall C. and Winter P. D.: Fetal and infant growth and impaired glucose tolerance at age 64. *Br. Med. J.* **303** (1991) 1019–1022.
36. Barker D. J. P., Osmond C., Golding J., Kuh D. and Wadsworth M. E. J.: Growth *in utero*, blood pressure in childhood and adult life, and mortality from cardiovascular disease. *Br. Med. J.* **298** (1989) 564–567.
37. Barker D. J. P., Bull A. R., Osmond C. and Simmonds S. J.: Fetal and placental size and risk of hypertension in adult life. *Br. Med. J.* **301** (1990) 259–262.

38. Osinski P. A.: Steroid 11β-ol dehydrogenase in human placenta. *Nature* **187** (1960) 777.
39. Lopez Bernal A., Flint A. P. F., Anderson A. B. M. and Turnbull A. C.: 11β-Hydroxysteroid dehydrogenase activity in human placenta and decidua. *J. Steroid Biochem.* **13** (1980) 1081–1087.
40. Beitins I. Z., Bayard F., Ances I. G., Kowarski A. and Migeon C. J.: The metabolic clearance rate, blood production, interconversion and transplacental passage of cortisol and cortisone in pregnancy near term. *Pediat. Res.* **7** (1973) 509–519.
41. Benediktsson R., Seckl J. R. and Edwards C. R. W.: Placental 11β-hydroxysteroid dehydrogenase: Relationship with fetal and placental size at term. *74th Meet. Am. Endocrine Soc.* San Antonio, TX (1992) Abstr. 622.
42. Csaba G.: Phylogeny and ontogeny of hormone receptors: the selection theory of receptor formation and hormonal imprinting. *Biol. Rev.* **55** (1980) 47–63.

Journal of Human Hypertension (1994) **8**, 371–375

Apparent mineralocorticoid excess

Rafn Benediktsson and Christopher R.W. Edwards
University Department of Medicine, Western General Hospital, Edinburgh EH4 2XU, UK

Summary:

In 1979, Ulick and New first coined the term Apparent Mineralocorticoid Excess (AME) for a syndrome of hypertension, hypokalaemia, suppressed renin–angiotensin–aldosterone axis and raised urinary ratio of 11beta-hydroxy to 11-oxo metabolites of cortisol (suggesting a failure of conversion of cortisol to cortisone). In retrospect, the first case was described in 1974 and since then over 20 children have been reported worldwide but only one adult patient. The enzyme 11beta-hydroxysteroid dehydrogenase (11beta-OHSD) confers aldosterone specificity on intrinsically nonspecific kidney mineralocorticoid receptors by converting the active glucocorticoid cortisol to its inactive 11-oxo form (cortisone). Patients with AME have a deficiency of this enzyme which allows physiological levels of cortisol to flood mineralocorticoid receptors. Dexamethasone, by suppressing adrenal cortisol production, reverts the biochemistry but not usually the BP to normal. Liquorice inhibits 11beta-OHSD by virtue of its active ingredient glycyrrhetinic acid, resulting in an identical clinical picture. Renal 11beta-OHSD is the protagonist in AME but this enzyme is found in many other tissues including liver, placenta and vasculature, and one-third of essential hypertensives have deficient 11beta-OHSD. The placental isoform is thought to be the main barrier to maternal glucocorticoids reaching the fetus. The lowest rat placental 11beta-OHSD activity is found in the largest placentas corresponding to the smallest fetuses (presumably exposed to the highest glucocorticoid levels). This is the group which in humans are most at risk of developing hypertension. In support of the hypothesis that glucocorticoid excess *in utero* imprints patterns leading to hypertension in adults is our observation that treatment of pregnant rats with dexamethasone leads to the development of hypertension in adult offspring.

Introduction

When presented with a patient suffering from hypertension in conjunction with hypokalaemia, aldosterone excess immediately springs to mind. Over the past two decades, however, a rare syndrome of hypertension with hypokalaemia and, intringuingly, suppressed plasma renin activity and aldosterone has emerged. More than 20 children with this disorder have now been diagnosed, distinguished from Liddle's syndrome by failure of the latter to respond to spironolactone. Other disorders like congenital adrenal hyperplasia and primary glucocorticoid resistance are distinguished from AME by virtue of raised 11-deoxycorticosterone (DOC) in these conditions. Clinical features in addition to hypertension in children with AME are short stature, failure to thrive, polyuria and polydipsia.

Although in retrospect the first case[1] was described in 1974 it was not until New and colleagues described similar cases[2,3] that the term Apparent Mineralocorticoid Excess (AME) was coined for this disorder. The term 'apparent' was appropriate, as intensive search for a hitherto unknown mineralocorticoid steroid was fruitless and DOC levels were not raised. The salient abnormality in this syndrome is a defect in the peripheral metabolism of the physiological glucocorticoid cortisol. Thus urinary 11beta-hydroxy metabolites of cortisol were raised in comparison with the 11-oxo metabolites (cortisone metabolites) indicating impaired conversion of the active glucocorticoid cortisol to its inactive counterpart cortisone. The half-life of cortisol was thus prolonged, estimated by conversion of 11alpha^{3}H-cortisol to unlabelled cortisone and tritiated water indicating impaired oxidation of the 11-hydroxyl group. Cortisol levels in peripheral blood were normal, however, accomplished by reduced adrenal secretion rate via hypothalamic/pituitary feedback.

These children responded to pituitary–adrenal suppression by administration of the pure glucocorticoid dexamethasone. Treatment with hydrocortisone aggravated the condition leading to the suggestion that cortisol was the mineralocorticoid involved,[4] but the exact mechanism was not explained until the first adult case was described in Edinburgh.

11beta-Hydroxysteroid dehydrogenase

The enzyme 11beta-hydroxysteroid dehydrogenase (11beta-OHSD) was first described in the placenta[5]

Correspondence: R. Benediktsson, Cand Met et Chir

in 1960 but has since been found in many tissues. This enzyme is an oxidoreductase complex catalysing the interconversion of active cortisol and inactive cortisone.

Ulick and New[2,3] had described an abnormality of the dehydrogenase component of 11beta-OHSD in the children with AME but the pivotal role in the pathogenesis of the syndrome remained obscure until a 20 year old male presented with severe hypertension, hypokalaemia and suppression of the renin–angiotensin–aldosterone axis.[6] The diagnosis of AME was made, based on metabolic balance studies and the urinary steroid metabolite profile along with a prolonged half-life of 11alpha^{3}H-cortisol confirming the impaired inactivation of cortisol. These studies showed that the hypertension and biochemical abnormalities responded to pituitary–adrenal suppression with dexamethasone and on oral hydrocortisone supplementation (or dexamethasone withdrawal) the syndrome could be reproduced with kaliuresis, sodium retention with corresponding changes in the plasma electrolytes and suppression of the renin–angiotensin–aldosterone axis. Concomitantly the patient's weight rose and there was an increase in his BP.

The liquorice connection

Key to understanding the pathogenesis of this syndrome was the recognition of the similarities between the type of hypertension induced by liquorice and the syndrome of AME. It had been known for many years that abuse of liquorice caused classical mineralocorticoid hypertension.[7] Suggested explanations included binding of glycyrrhetinic acid (the active component of liquorice) to the kidney mineralocorticoid receptor[8] but two facts contradicted this. First, the affinity of the mineralocorticoid receptor for glycyrrhetinic acid was far lower than that for aldosterone and second, some elegant studies in both humans[9] and animals[10] had shown that an intact adrenal gland was necessary for liquorice to exert its action. In fact, oral cortisone was able to restore the effect of glycyrrhetinic acid in patients with Addison's disease[9] and dexamethasone produced natriuresis in subjects taking glycyrrhetinic acid.[11]

Further studies reproduced AME in subjects taking liquorice, confirmed by raised urinary excretion of 11beta-hydroxy metabolite of cortisol in comparison to 11-oxo metabolites (cortisone metabolites), sodium retention, kaliuresis and a prolonged half-life of 11alpha^{3}H-cortisol with suppression of the renin–angiotensin–aldosterone axis.[12] It therefore seemed that liquorice abuse resulted in an acquired form of AME.

To clinch the site of the defect Edwards and Stewart[12] looked at the effect of glycyrrhetinic acid on binding of glucocorticoids to renal receptors.[13] This was carried out in the rat where the physiological glucocorticoid is corticosterone but not cortisol. It was shown by autoradiography that inhibition of 11beta-OHSD in rat kidney with glycyrrhizic acid allowed ^{3}H-corticosterone to bind to aldosterone binding sites whereas none bound in the absence of the 11beta-OHSD inhibitor.

In vitro work with purified type 1 mineralocorticoid receptor[14] as well as cloning and expression of the human type 1 receptor[15] has since shown it to have similar affinities for cortisol and aldosterone lending plausibility to the hypothesis that *in vivo* an additional factor was necessary to ensure tissue mineralocorticoid specificity, namely 11beta-OHSD. This hypothesis is obviously of paramount importance in a biological sense, challenging the simplistic view that receptor activation depended solely on ligand–receptor interaction.

Variants of the AME syndrome

The above description relates to what is now known as type 1 AME, being a defect in the dehydrogenase component of 11beta-OHSD but not the oxidase component. This is supported by finding a normal increase in plasma cortisol following oral administration of cortisone in addition to the characteristic urinary steroid metabolite profile and a prolonged half-life of 11alpha^{3}H-cortisol.

Recently Ulick and colleagues described three cases of what has become known as type 2 AME.[16,17] These patients have an identical picture of low renin hypertension with hypokalaemic alkalosis and suppressed aldosterone and DOC but a normal ratio of urinary metabolites of cortisol/cortisone. There is evidence for abnormal steroid metabolism beyond 11beta-OHSD and until further studies have been carried out in these individuals the precise defect underlying this particular syndrome has to be considered unknown. However, administration of carbenoxolone, the hemisuccinate derivative of glycyrrhetinic acid which inhibits both the dehydrogenase and reductase component of 11beta-OHSD, produces a similar state in humans.[18]

Variants of 11beta-hydroxysteroid dehydrogenase

Knowledge about 11beta-OHSD is currently accumulating with amazing speed, new discoveries calling for a wider appraisal of its role. In the liver the enzyme's prevailing direction appears to be opposite to that in the kidney. Similarly, different tissues exhibit different activities, show different cofactor preferences and a body of knowledge is accumulating about regulation of the enzyme by various influences in different tissues. Tissue specific differences in 11beta-OHSD may not only be the result of variations in the intracellular environment or post-translational modifications but probably represents different species of the enzyme.

The initial idea that the enzyme protected the renal mineralocorticoid receptor in a paracrine fashion stemmed from the observation of positive immunostaining for the enzyme in the proximal tubules, upstream from the classical mineralocorticoid receptor sites.[13] Later studies however,[19,20] confirmed bioactivity in the distal tubules supporting

the more plausible autocrine hypothesis (i.e. 11beta-OHSD regulates access to receptors within the same cell) and recently multiple mRNA species have been identified in the kidney.[21] Recent studies have identified at least two renal isoforms of 11beta-OHSD.[22–24]

Finally, there is not only evidence now for multiple isoforms of 11beta-OHSD but work from our laboratory[25] has shown the human placenta to contain 11beta-OHSD of a different size to the liver isoform having different cofactor preferences and kinetic parameters. The differences indicate that it is possible that the 'liver-type' 11beta-OHSD and 'placental-type' 11beta-OHSD are the products of two separate genes.

Role for extrarenal 11beta-OHSD

Although the bulk of plasma cortisone derives from the kidney as we have shown[26,27] and another pathogenic role for the renal enzyme has been demonstrated in the ectopic ACTH syndrome,[27] the wide distribution of 11beta-OHSD in mammalian tissue indicates the probability of a more widespread role. In the ectopic ACTH syndrome, ACTH is thought to directly inhibit the kidney enzyme as assessed by plasma cortisol/cortisone ratios in individuals with Cushing's syndrome and in healthy volunteers infused with cortisol or ACTH resulting in a similar elevation of plasma cortisol.

Outside the kidney, one of the earlier observations from our laboratory was the potentiation of action of topically applied glucocorticoids by co-application of glycyrrhetinic acid.[28] The skin vasoconstrictor assay used to compare glucocorticoid potency is simply an estimate of dermal blood flow and this, along with the observation in AME that although sodium retention occurs in the acute phase BP increase occurs only after a lag period,[6] prompted us to look for other sites of 11beta-OHSD expression which might play part in BP regulation. Another stimulating observation was also at hand, namely that dexamethasone suppresses hypertension in a proportion of what otherwise would be considered patients with essential hypertension.[29,30]

We looked at rat vasculature[31] and found positive immunostaining and mRNA expression for 11beta-OHSD in vascular smooth muscle. The density of 11beta-OHSD was found to be higher in the appropriate locations, i.e. resistance vessels. It was also well known from animal experiments (for review see ref. 32) that glucocorticoids affect vascular responses to a variety of agents. When these observations were followed-up in humans *in vivo*, healthy volunteers consuming liquorice had increased dermal vasoconstriction to cortisol, the dermal vasoconstriction being still more intense in a subject with AME type 1.[33] Individuals with essential hypertension similarly had more intense dermal vasoconstriction in response to topically applied glucocorticoid than controls and about 30% of hypertensive individuals have prolonged half-life of 11alpha^{3}H-cortisol.[34]

Recent epidemiological data has implicated early development in the pathogenesis of ischaemic heart disease.[35] These data are especially striking for BP where the strongest correlate for elevated childhood[36] and adult[37] BP was the combination of low birthweight and a large placenta. Of particular importance is the fact that this is not only observed at the extremes of weights but a continuous spectrum exists. On analysing all the currently available data it was concluded that the fetal environment (rather than infant development) was the most important determinant.[38]

How this might come about is by no means clear although suggestions of malnutrition being the most important factor have been put forward. This hypothesis is still surrounded with controversy and we have looked for an alternative. It may be that 11beta-OHSD again plays a decisive role. The placental enzyme was the first isoform to be discovered[5] and has been believed to be the main protecting mechanism against the well-known growth inhibiting effects of glucocorticoids on the fetus.[39] Ontogeny and regulation by sex steroids of the placental enzyme has then been thought to ensure correct timing of development of the fetal hypothalamic–pituitary–adrenal axis in baboons[40] and perhaps other fetal developmental aspects in other species.[41]

For our hypothesis of a deficiency in placental 11beta-OHSD allowing increased access of physiological maternal glucocorticoids to the fetus causing fetal growth impairment, it was crucial to discover that the unusual pattern of a low birthweight with high placental weight had been observed in rats with streptozotocin-induced diabetes mellitus[42,43] which have markedly increased glucocorticoid levels.[44]

We tested this hypothesis[45] by examining rat placental 11beta-OHSD activity *in vitro*, correlating it with birth- and placental weights. We found a positive correlation of enzyme activity with birthweight and a negative correlation with placental weight. Both were highly significant and indeed the largest placentas which had the lowest enzyme activity corresponded to the smallest fetuses, which is the group in humans most at risk for hypertension. Several explanations can be thought of to explain how glucocorticoid excess *in utero* might lead to adult disease. One of them is the phenomenon of imprinting (or programming) which is well recognised for steroid hormones[46] as well as for vasoactive substances like vasopressin.[47] Effects on the brain leading to deranged control of development of other systems is also a possibility supported by observations on neonatal handling (stress) of rats which modifies hippocampal glucocorticoid receptor mRNA expression.[48]

To look at whether programming of hypertension by glucocorticoid excess *in utero* did take place, we measured BP in adult offspring of rats receiving dexamethasone while pregnant.[45] This synthetic glucocorticoid escapes metabolism by 11beta-OHSD by virtue of 9alpha-fluoro substitution ensuring fetal exposure. No effects were observed on litter size, resorption rate or frequency of offspring malformation when compared with a control group

receiving vehicle only. When the BP was measured in early adulthood we observed a significantly raised SBP in both males and females when compared with controls.

We have now obtained further evidence indicating a wider role for 11beta-OHSD in protecting the developing individual from an earlier stage as the enzyme is found in the rat ovary where it seems to concentrate in the oocyte itself.[49]

Conclusion

The discovery of the role of 11beta-OHSD in the pathogenesis of the very rare syndrome of AME has led to further investigations on this widely distributed enzyme. We now have indications for the existence of more than one type of this enzyme which dictates the effects of glucocorticoids in a tissue-specific manner. A potential for a role in more common disorders like essential hypertension and ischaemic heart disease is emerging.

References

1. Werder, E., Zachmann, M. & Vollmin, J.A. Unusual steroid excretion in a child with low renin hypertension. *Research in Steroids* 1974, **6**: 385–389.
2. New, M.I., Levine, L.S., Biglieri, E.G., Pariera, J. & Ulick, S. Evidence for an unidentified steroid in a child with apparent mineralocorticoid hypertension. *J Clin Endocrinol Metab* 1977, **44**: 924–933.
3. Ulick, S., Levine, L.S., Gunczler, P. *et al.* A syndrome of apparent mineralocorticoid excess associated with defects in the peripheral metabolism of cortisol. *J Clin Endocrinol Metab* 1979, **49**: 757–764.
4. Oberfield, S.E., Levine, L.S., Carey, R.M., Grieg, F., Ulick, S. & New, M.I. Metabolic and blood pressure responses to hydrocortisone in the syndrome of apparent mineralocorticoid excess. *J Clin Endocrinol Metab* 1983, **56**: 332–339.
5. Osinski, P.A. Steroid 11β-ol dehydrogenase in human placenta. *Nature* 1960, **187**: 777.
6. Stewart, P.M., Corrie, J.E.T., Shackleton, C.H.L. & Edwards, C.R.W. Syndrome of apparent mineralocorticoid excess. A defect in the cortisol-cortisone shuttle. *J Clin Invest* 1988, **82**: 340–349.
7. Salassa, R.M., Mattox, V.R. & Rosevear, J.W. Inhibition of mineralocorticoid activity of liquorice by spironolactone. *J Clin Endocrinol* 1962, **22**: 1156–1159.
8. Armanini, D., Karbowiak, I. & Funder, J.W. Affinity of liquorice derivatives for mineralocorticoid and glucocorticoid receptors. *Clin Endocrinol* 1983, **19**: 609–612.
9. Borst, J.G.G., Ten Holt, S.P. & Devries, L.A. Synergistic action of liquorice and cortisone in Addison's and Simmond's disease. *Lancet* 1953, **i**: 657–663.
10. Card, W.I., Mitchell, W., Strong, J.A. & Taylor, N.R.W. Effects of liquorice and its derivatives on salt and water metabolism. *Lancet* 1953, **i**: 663–668.
11. Hoefnagels, W.H.L. & Kloppenborg, P.W. Antimineralocorticoid effect of dexamethasone in subjects treated with glycyrrhetinic acid. *J Hypertens* 1983, **1** (Suppl. 2): 313–315.
12. Stewart, P.M., Wallace, A.M., Valentino, R., Burt, D., Shackleton, C.H.L. & Edwards, C.R.W. Mineralocorticoid activity of liquorice: 11-beta-hydroxysteroid dehydrogenase deficiency comes of age. *Lancet* 1987, **ii**: 821–823.
13. Edwards, C.R.W., Stewart, P.M., Burt, D. *et al.* Localisation of 11β-hydroxysteroid dehydrogenase-tissue specific protector of the mineralocorticoid receptor. *Lancet* 1988, **ii**: 986–989.
14. Krozowski, Z.S. & Funder, J.W. Renal mineralocorticoid receptors and hippocampal corticosterone-binding species have identical intrinsic steroid specificity. *Proc Nat Acad Sci USA* 1983, **80**: 6056–6060.
15. Arriza, J.L., Weinberger, C. & Cerelli, G. Cloning of human mineralocorticoid receptor complementary DNA; structural and functional kinship with the glucocorticoid receptor. *Science* 1987, **237**: 268–275.
16. Ulick, S., Chan, C.K., Rao, K.N., Edassery, J. & Mantero, F. A new form of the syndrome of apparent mineralocorticoid excess. *J Steroid Biochem* 1989, **32**: 209–212.
17. Ulick, S., Tedde, R. & Mantero, F. Pathogenesis of the type 2 variant of the syndrome of apparent mineralocorticoid excess. *J Clin Endrocrinol Metab* 1990, **70**: 200–206.
18. Stewart, P.M., Wallace, A.M., Atherden, S.M., Shearing, C.H. & Edwards, C.R.W. Mineralocorticoid activity of carbenoxolone: contrasting effects of carbenoxolone and liquorice on 11β-hydroxysteroid dehydrogenase activity in man. *Clin Sci* 1990, **78**: 49–54.
19. Náray-Fejes-Tóth, A., Watlington, C.O. & Fejes-Tóth, G. 11 Beta-hydroxysteroid dehydrogenase activity in the renal target cells of aldosterone. *Endocrinology* 1991, **129**: 17–21.
20. Bonvalet, J.P., Doignon, I., Blot-Chabaud, M., Pradelles, P. & Farman, N. Distribution of 11 beta-hydroxysteroid dehydrogenase along the rabbit nephron. *J Clin Invest* 1990, **86**: 832–837.
21. Krozowski, Z.S., Stuchbery, S., White, P.C., Monder, C. & Funder, J.W. Characterization of 11 beta-hydroxysteroid dehydrogenase gene expression: identification of multiple unique forms of messenger ribonucleic acid in the rate kidney. *Endocrinology* 1990, **127**: 3009–3013.
22. Walker, B.R., Campbell, J.C., Williams, B.C. & Edwards, C.R.W. Tissue-specific distribution of the NAD(+)-dependent isoform of 11 beta-hydroxysteroid dehydrogenase. *Endocrinology* 1992, **131**: 970–972.
23. Krozowski, Z.S., Obeyesekere, V., Smith, R. & Mercer, W.R. Tissue-specific expression of an 11 beta-hydroxysteroid dehydrogenase with a truncated N-terminal domain. A potential mechanism for differential intracellular localization within mineralocorticoid target cells. *J Biol Chem* 1992, **267**: 2569–2574.
24. Mercer, W.R. & Krozowski, Z.S. Localization of an 11 beta-hydroxysteroid dehydrogenase activity to the distal nephron. Evidence for the existence of two species of dehydrogenase in the rat kidney. *Endocrinology* 1992, **130**: 540–543.
25. Brown, R.W., Chapman, K.E., Edwards, C.R.W. & Seckl, J.R. Human placental 11β-hydroxysteroid dehydrogenase: evidence for and partial purification of a distinct NAD-dependent isoform. *Endocrinology* 1993, **132**: 2614–2621.
26. Whitworth, J.A., Stewart, P.M., Burt, D., Atherden, S.M. & Edwards, C.R.W. The kidney is the major site of cortisone production in man. *Clin Endocrinol* 1989, **31**: 355–361.
27. Walker, B.R., Campbell, J.C., Fraser, R., Stewart, P.M. & Edwards, C.R.W. Mineralocorticoid excess and inhibition of 11β-hydroxysteroid dehydrogenase in

patients with ectopic ACTH syndrome. *Clin Endocrinol* 1992, **37**: 483–492.
28. Teelucksingh, S., Mackie, A.D.R., Burt, D., McIntyre, M.A., Brett, L.P. & Edwards, C.R.W. Potentiation of hydrocortisone activity in skin by glycyrrhetinic acid. *Lancet* 1990, **335**: 1060–1063.
29. Hamilton, B.P., Zadik, Z., Edwin, C.M., Hamilton, J.H. & Kowarski, A.A. Effect of adrenal suppression with dexamethasone in essential hypertension. *J Clin Endocrinol Metab* 1979, **48**: 848–853.
30. Whitworth, J.A., Gordon, D., McLachlan-Troup, N., Scoggins, B.A. & Moulds, R.W.F. Dexamethasone suppression in essential hypertension: effects on cortisol and blood pressure. *Clin Exp Hypertens – Part A, Theory & Practice* 1989, **II**: 323–335.
31. Walker, B.R., Yau, J.L.W., Brett, L.P. *et al.* 11 Beta-hydroxysteroid dehydrogenase in vascular smooth muscle and heart: implications for cardiovascular responses to glucocorticoids. *Endocrinology* 1991, **129**: 3305–3312.
32. Walker, B.R. & Williams, B.C. Corticosteroids and vascular tone: mapping the messenger maze. *Clin Sci* 1992, **82**: 597–605.
33. Walker, B.R., Connacher, A.A., Webb, D.J. & Edwards, C.R.W. Glucocorticoids and blood pressure: a role for the cortisol/cortisone shuttle in the control of vascular tone in man. *Clin Sci* 1992, **83**: 171–178.
34. Walker, B.R., Stewart, P.M., Padfield, P.L. & Edwards, C.R.W. Increased vascular sensitivity to glucocorticoids in essential hypertension: 11β-hydroxysteroid dehydrogenase deficiency revisited. *J Hypertens* 1991, **9**: 1082–1083.
35. Barker, D.J.P., Osmond, C., Simmonds, S.J. & Wield, G.A. The relation of head size and thinness at birth to death from cardiovascular disease in adult life. *Br Med J* 1993, **306**, 422–426.
36. Law, C.M., Barker, D.J.P., Bull, A.R. & Osmond, C. Maternal and fetal influences on blood pressure. *Arch Dis Child* 1991, **66**: 1291–1295.
37. Barker, D.J.P, Bull, A.R., Osmond, C. & Simmonds, S.J. Fetal and placental size and risk of hypertension in adult life. *Br Med J* 1990, **301**: 259–262.
38. Law, C.M., de Swiet, M., Osmond, C *et al.* Initiation of hypertension in *utero* and its amplification throughout life. *Br Med J* 1993, **306**: 24–27.
39. Beitins, I.Z., Bayard, F., Ances, I.G., Kowarski, A. & Migeon, C.J. The metabolic clearance rate, blood production, interconversion and transplacental passage of cortisol and cortisone in pregnancy near term. *Pediat Res* 1973, **7**: 509–519.
40. Baggia, S., Albrecht, E.D. & Pepe, G.J. Regulation of 11 beta-hydroxysteroid dehydrogenase activity in the baboon placenta by estrogen. *Endocrinology* 1990, **126**: 2742–2748.
41. Murphy, B.E.P. Ontogeny of cortisol-cortisone interconversion in human tissues: a role for cortisone in human fetal development. *J Steroid Biochem* 1981, **14**: 811–817.
42. Robinson, J., Canavan, J.P., El Haj, A.J. & Goldspink, D.F. Maternal diabetes in rats. I. Effects on placental growth and protein turnover. *Diabetes* 1988, **37**: 1665–1670.
43. Canavan, J.P. & Goldspink, D.F. Maternal diabetes in rats. II. Effects on fetal growth and protein turnover. *Diabetes* 1988, **37**: 1671–1683.
44. Heller, C.L., Weisenberg, L.S., Ortí, E. & de Nicola, A.F. Steps of glucocorticoid action in normal and diabetic rat placenta. *J Steroid Biochem* 1988, **31**: 119–123.
45. Benediktsson, R., Lindsay, R.S., Noble, J., Seckl, J.R. & Edwards, C.R.W. Glucocorticoid exposure *in utero*: new model for adult hypertension. *Lancet* 1993, **341**: 339–341.
46. Gustafsson, G.A. & Stenberg, A. Irreversible androgenic programming at birth of microsomal and soluble rat liver enzymes active on 4-androstene-3, 17-dione and 5-alpha, androstane-3-alpha, 17-beta-diol. *J Biol Chem* 1974, **249**: 711–718.
47. Csaba, G., Rónai, A., László, V., Darvas, Z. & Berzétei, I. Amplification of hormone receptors by neonatal oxytocin and vasopressin treatment. *Horm Metab Res* 1980, **12**, 28–31.
48. LaRocque, S., O'Donnell, D., Gianoulakis, C., Seckl, J.R. & Meaney, M.J. Postnatal handling in the rat alters hippocampal glucocorticoid receptor gene expression. *Soc Neurosci Abstr* 1992, A204.7.
49. Benediktsson, R., Yau, J.L.W., Low, S. *et al.* 11 Beta-hydroxysteroid dehydrogenase in the rat ovary: high expression in the oocyte. *J Endocrinol* 1992, **135**: 53–58.